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STUDIES ON THE ESTIMATION, BIOAVAILABILITY AND INTERACTIONS OF SOME SALICYLATE PREPARATIONS

by

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A THESIS
SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH
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THE UNIVERSITY OF ALBERTA
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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research, for acceptance, a thesis entitled STUDIES ON THE ESTIMATION, BIOAVAILABILITY AND INTERACTIONS OF SOME SALICYLATE PREPARATIONS submitted by Leo J. Walter in partial fulfilment of the requirements for the degree of Master of Science in Biopharmacy.

This thesis is dedicated to my wife,
Wendy, whose patience and understanding
made the endeavor possible.

ABSTRACT

A method for the simultaneous gas-liquid determination of salicylic acid and acetylsalicylic acid in plasma has been developed. The method involved the use of fluoride and cooling to inhibit the hydrolysis of acetylsalicylic acid in whole blood. One ml portions of plasma separated by centrifugation were acidified with ten percent potassium bisulfate solution and extracted into chloroform. The chloroform was recovered, internal standards added, and the solution containing the salicylates and internal standards evaporated to dryness on a flash evaporator at 20°. The respective trimethylsilyl ether and/or trimethylsilyl ester derivatives of the salicylates were synthesized by reaction with hexamethyldisilazane in acetone at room temperature for fifty minutes. The reaction mixture was chromatographed on a column of 3% OV-25 on 60/80 mesh Chromosorb G (regular), and eluted isothermally at an oven temperature of 160° and a carrier gas flow rate of 55 ml min^{-1}. The area ratios of the salicylate derivatives to internal standards and the respective calibration curves were used to determine the amount of salicylic acid and acetylsalicylic acid present in each one ml sample of plasma.

This methodology was then employed to compare the *in vivo* bioavailabilities of various formulations of acetylsalicylic acid in arthritic in-patients. Three enteric-coated, one buffered and one 'plain' brand were investigated.

The same batch of tablets were then subjected to the USP Disintegration Test as an *in vitro* bioavailability test. Among the enteric-coated formulations a correlation was noted between the time required to disintegrate in Intestinal Fluid, T.S. and the percent availability in four hours. Half-lives and percent absorbed versus time plots were determined for each formulation.

The methodology was extended to investigate a suspected interaction of ascorbic acid and acetylsalicylic acid at the absorption level. This interaction was investigated in rabbits and was not found to be significant.

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INTRODUCTION

I. Survey of Published Methodology

Numerous methods have been described in the literature for the determination of salicylic acid (SA), and acetylsalicylic acid (ASA), in pharmaceuticals and in biological media. Of these published techniques, surprisingly few have been developed for the simultaneous and direct determination of ASA and SA in a single sample.

These techniques may be classified on the basis of their methodology into five general divisions.

The first division is that of the colorimetric estimations. These include, complexation with ferric ion (Brodie Udenfriend and Coburn, 1944; Keller, 1947; Peters, 1947; Miller and Whitehead, 1949; Trinder, 1954; Cotty, Zurzola, Beezley and Rodgers, 1965; Furman and Finberg, 1967; Burston, 1969; Cid, Delporte and Jaminet, 1971), diazotization with p-nitroaniline and nitrous acid (Moss, 1952), use of Folin and Ciocalteaus phenol reagent (Weichselbaum and Shapiro, 1945; Smith and Talbot, 1950), complexation with cupric ion in nitrous acid (Sherman and Gross, 1911; Mallick and Rehmann, 1945; Reid, 1948), and estimation as nitro derivatives (Volterra and Jacobs, 1947). Colorimetric assays are generally quite non-specific. Plasma blank values are often high, and equivalents of up to 240 μg SA ml^{-1} have been reported (Weichselbaum and Shapiro, 1945). Common plasma constituents such as salicyluric acid (Brodie, Udenfriend and Coburn, 1944; Smith and Talbot, 1950), gentisic acid, uric acid, tyrosine, and tryptophan (Smith and

Talbot, 1950) interfere with colorimetric assays. This entire group of assays determines SA only, and in order to estimate ASA, hydrolysis to SA and another determination are required. The difference in the two SA concentrations is assumed to be due to ASA.

Among the advantages enjoyed by colorimetric estimations is the fact that small amounts of sample, usually one ml or less, are required. The procedures are also relatively rapid and a single determination requires as little as five minutes (Trinder, 1954).

The second analytical group is that of the ultraviolet estimations. By these techniques either SA alone, or SA plus ASA may be determined. SA has been estimated, either in combination with ASA (Reed and Davis, 1965) or individually following a physical separation step (Jones and Thatcher, 1951; Ungar, Damgaard and Wong, 1952; Levine and Weber, 1968; Guttman and Salomon, 1969). Both salicylates may be determined simultaneously by use of the pH-dependent shift in their individual absorption spectra (Williams, Linn and Zak, 1959; Clayton and Thiers, 1966; Routh, Shane, Arredondo and Paul, 1967). This hypsochromic shift technique results in some overlap of the absorption spectra of SA and ASA, and corrections are required for these spectral interferences (Clayton and Thiers, 1966; Routh, Shane, Arredondo and Paul, 1967). The hypsochromic shift technique also requires dual determinations, one at each of two pH's. The individual

determinations of SA are subject to interference from ASA, gentisic acid, and 2,6-dihydroxybenzoic acid (Ungar, Damgaard and Wong, 1952). This interference may be greatly reduced by using the pH-dependent shift method (Routh, Shane, Arredondo and Paul, 1967).

The third methodological division is the fluorometric group. By these techniques, ASA may be estimated directly (Miles and Schenk, 1970), or as is more often the case, either after hydrolysis to SA by the "difference method" (Øie and Frislid, 1970), or after a separation step followed by hydrolysis. Separation has been achieved by paper chromatography (Lange and Bell, 1966), by chemical reaction with ceric ammonium nitrate (Cotty and Ederma, 1966; Kanter and Horbaly, 1971), and by gel filtration (Lee, Thompkins and Spencer, 1968). By analogy to the colorimetric assays, fluorometric analyses are specific for SA only, which necessitates dual determinations, or a separation and hydrolysis procedure in order to estimate ASA. Another problem with fluorometric assays is the high and variable background fluorescence. The use of blank plasma from each individual subject minimizes this problem (Øie and Frislid, 1970). Fluorometric assays are relatively free of interference from substances such as salicylamide, salicyluric acid, and salicylic acid ether glucuronide, following separation (Cotty and Ederma, 1966; Kanter and Horbaly, 1971). Another advantage is that relatively small amounts of sample are

required. Micro-methods employing as little as 0.1 ml of blood have been developed (Cotty and Ederma, 1966; Øie and Frislid, 1970).

The fourth analytical group is that of the chromatographic estimations. Thin layer chromatography (Cummings and King, 1966; Morrison and Orr, 1966) and liquid chromatography have been employed (Stevenson and Burtiss, 1971); however, the majority of the quantitative chromatographic procedures used for salicylates involve gas liquid chromatography (GLC). Estimation of ASA and SA as the free acids by GLC (Hoffman and Mitchell, 1963; Nikelly, 1964; Dechene, Booth and Caughey, 1969) is difficult due to their low vapour pressure and the presence of polar functional groups which cause adsorption and tailing. Masking the functional groups by derivatization renders these molecules much less polar, more volatile, and consequently more amenable to GLC analysis. Among the derivatives which have been prepared quantitatively are the methyl ester- methyl ether derivatives (Crippen and Freimuth, 1964; Morris, Christian, Landolt and Hansen, 1970; Watson, Crescuolo and Matsui, 1971), and the trimethylsilyl (TMS) derivatives (Horii, Makita, Takeda, Tamura and Ohnishi, 1965; Blakely, 1966; Rowland and Riegelman, 1967; Rowland and Riegelman, 1967a; Mamer, Crawhall and Tjoa, 1970).

One advantage of GLC methods is that they permit the estimation of both compounds simultaneously (Nikelly, 1964;

Crippen and Freimuth, 1964; Morris, Christian, Landolt and Hansen, 1970; Watson, Crescuolo and Matsui, 1971). The estimations are also relatively rapid and may require as little as three minutes to estimate both salicylates (Morris, Christian, Landolt and Hansen, 1970).

The fifth group of analytical methods is a miscellaneous category of techniques which are used less frequently, for example, infrared spectrophotometry (Parke, Ribley, Kennedy and Hilty, 1951), nuclear magnetic resonance spectrometry (Winefordner and Latz, 1963; Miles and Schenk, 1970), and non-aqueous titrimetry (Lin, 1967; Fogg, Sausins and Smithson, 1970).

The most promising of these methods appear to be GLC estimations. The reasons for this are relatively straightforward. First of all, both SA and ASA may be determined directly and simultaneously in a single chromatographic run. Secondly, they are considerably less time consuming than the colorimetric, ultraviolet and fluorometric assays because of the elimination of the hydrolytic and separation steps, as well as the dual determination. Thirdly, they are also the most specific of the methods discussed. Even similar compounds, such as benzoic acid, m-hydroxybenzoic acid and p-hydroxybenzoic acid (Blakely, 1966); 2,5-dihydroxybenzoic acid, 2,3-dihydroxybenzoic acid, and 2,3,4-trihydroxybenzoic acid (Morris, Christian, Landolt and Hansen, 1970); and acetophenetidin (Hoffman and Mitchell,

1963) will exhibit different retention times from the salicylates, and will consequently not affect their estimation. Fourthly, GLC methods are quite sensitive and compare favourably with fluorometric procedures. For example, 0.4 μg ASA ml^{-1} may be determined in the presence of a vast excess of SA (Rowland and Riegelman, 1967a).

Although ASA has been estimated as the free acid by GLC (Hoffman and Mitchell, 1963; Dechene, Booth and Caughey, 1969), no evidence is presented by these authors for similar SA determinations. The GLC estimation of free ASA has been described as difficult (Nikelly, 1964) due to its low vapor pressure and the presence of the carboxylic acid functional group which interacts with the solid column support to cause adsorption and tailing. Similarly, SA with two polar functional groups should undergo greater adsorption and be even more difficult to chromatograph.

To reduce the polarity of the free salicylates, and render them more amenable to GLC analysis, the functional groups may be masked in derivatives. Some success has been achieved in the analysis of SA and ASA as their respective methyl ester- methyl ether derivatives (Crippen and Freimuth, 1964; Morris, Christian, Landolt and Hansen, 1970; Watson, Crescuolo and Matsui, 1971). However, these procedures have distinct disadvantages:

a) Methylation reactions may be tedious using BF_3/methanol because of a refluxing step (Crippen

and Freimuth, 1964).

b) Alternate syntheses of the methyl derivatives involve the generation and use of diazomethane, both of which may be hazardous (Morris, Christian, Landolt and Hansen, 1970; Watson, Crescuolo and Matsui, 1971).

Another derivative which has been widely employed in the GLC analysis of these and related compounds are the TMS ester- TMS ether derivatives (Horii, Makita, Takeda, Tamura and Ohnishi, 1965; Blakely, 1966; Rowland and Riegelman, 1967; Rowland and Riegelman, 1967a). None of these procedures describes the use of TMS derivatives for the simultaneous estimation of both SA and ASA in biological samples. With these problems in mind it was decided to develop a method for the simultaneous estimation of the TMS derivatives of both salicylates by GLC in biological samples. TMS derivatives were selected over methyl derivatives because their reagents are relatively convenient to work with, there is less risk involved in their use, and they are available as such and need not be prepared prior to use.

II. Review of Literature on Salicylate Absorption

Orally administered salicylates are rapidly absorbed. Peak blood levels have been obtained after thirty minutes with a solution of ASA, and after sixty minutes with tablets (Leonards, 1963; Levy, 1964; Pütter and Bauer, 1970; Rowland, Riegelman, Harris and Sholkoff, 1972). Absorption occurs to some extent from the stomach (Hogben, Shanker, Tocco and Brodie, 1957; Shanker, Shore, Brodie and Hogben, 1957; Weikel and Lish, 1959; Doluisio, Billups, Dittert, Sugita and Swintosky, 1969), but mainly from the small intestine (Shanker, Tocco, Brodie and Hogben, 1958; Weikel and Lish, 1959; Doluisio, Billups, Dittert, Sugita and Swintosky, 1969).

The predominant mechanism of salicylate absorption is a physical process of passive diffusion of nondissociated molecules across the gastrointestinal membranes (Shanker, Shore, Brodie and Hogben, 1957; Shanker, Tocco, Brodie and Hogben, 1958; Hogben, Tocco, Brodie and Shanker, 1959). This 'pH Partition Hypothesis' states that the rate of absorption of drugs depends upon the concentration of non-ionized molecules, and their lipid solubility (Goodman and Gilman, 1970). At the low pH of the stomach, salicylates are expected to exist primarily in the unionized form. Consequently, their lipid solubility is high, and they are expected to be absorbed rapidly.

On the other hand, the pH of the intestine is much higher than that of the stomach. This pH virtually ensures

complete ionization of the salicylates, and greatly reduces their lipid solubility. Theoretically then, their absorption from the intestine should be insignificant. The rapid intestinal absorption observed may be rationalized in terms of the extremely large mucosal area of the small intestine, which apparently reduces the need for a large fraction of the drug to be present in the unionized form. Large numbers of folds of Kerkring, villi and microvilli in the small intestine have been estimated to increase the surface area of the small intestine approximately six hundred times, relative to a cylinder of comparable dimensions (Wilson, 1962). The net result is that the intestine is the major site of salicylate absorption (Hogben, 1960; Doluisio, Billups, Dittert, Sugita and Swintosky, 1969).

The pH Partition Hypothesis has been criticized for not adequately explaining some observed intestine-to-plasma drug concentrations. Experimental results suggested that the minimum pka value consistent with rapid absorption of a weak acid is approximately 3.0, while the highest pka compatible with the absorption of a weak base is 8.0. Assuming the pH of the intestinal fluid to remain relatively constant at 6.5, the minimal ratio of nonionized to ionized molecules required for rapid absorption of weak acids is 1:3000, and 1:30 for weak bases. This large difference implies that the intestinal barrier is less permeable to the ionized form of weak bases than to the ionized form of

weak acids. This differential permeability would not be consistent with the concept of a passive lipoidal-sieve membrane. In order to achieve quantitative conformity, it has been postulated that a 'microclimate' exists at the epithelial surface of the small intestine, which has a virtual pH of about 5.3, regardless of the pH of the luminal fluid (Shanker, Tocco, Brodie and Hogben, 1958; Hogben, Tocco, Brodie and Shanker, 1959; Hogben, 1960; Doluisio, Billups, Dittert, Sugita and Swintosky, 1969).

It has been demonstrated, by an in situ technique, that the absorption of ASA and SA from the rat stomach is increased in an acid medium at pH 1.0 compared to a pH of 8.0 (Shanker, Shore, Brodie and Hogben, 1957). These observations have been extended by human studies (Cooke and Hunt, 1969; Cooke and Hunt, 1970), where it was observed that unbuffered ASA is absorbed from the stomach at approximately ten times the rate of buffered ASA. Buffered ASA is emptied more rapidly from the stomach than is unbuffered ASA (Hunt, 1963; Cooke and Hunt, 1970); in fact, unbuffered ASA has been shown to reduce the rate of gastric emptying (Weikel and Lish, 1959). These observations are consistent with the hypothesis that absorption is a function of the concentration of nonionized molecules.

The absorption of salicylates follows apparent first order kinetics (Levy and Hollister, 1964; Doluisio, Billups, Dittert, Sugita and Swintosky, 1969; Doluisio, Tan, Billups

and Diamond, 1969), with a half-life of absorption ranging from 4.5 to 16 minutes (Rowland, Riegelman, Harris and Sholkoff, 1972). The concentration gradient across the mucosa is maintained by the normal circulation of blood through the mucosa and submucosa.

Numerous publications have reported significantly higher blood levels of salicylate from buffered versus unbuffered preparations (Paul, Dryer and Routh, 1950; Carlo, Cambosos, Feeney and Smith, 1955; Truitt and Morgan, 1962; Leonards, 1963; Cotty, Zurzola, Beezley and Rodgers, 1965). These findings seem to refute the theory that passive diffusion of unionized molecules is responsible for absorption. If this effect is localized in the stomach, one possible explanation is that the continuous secretion of hydrogen ions maintains a much lower pH at the site of absorption than in the bulk fluid of the gastric pouch (Hogben, 1960). Unfortunately, these studies used blood levels of salicylate as the index of absorption, and could therefore not provide any information regarding the sites of absorption, nor of the effects of buffers at these sites. A more plausible explanation is that the effect of the buffers is to produce more rapid transfer of the gastric contents to the intestine, the major site of salicylate absorption.

Salicylate absorption from enteric-coated preparations is designed to occur exclusively in the small intestine.

In practice, however, they are poorly and incompletely absorbed (Levy and Hollister, 1964), and produce lower salicylate levels than do uncoated formulations (Hollister and Kanter, 1964; Leonards and Levy, 1965), with significant plasma levels not being attained until six or more hours after administration. Considerable inter-subject variation has been reported with enteric-coated preparations (Blythe, Grass and MacDonell, 1959; Hollister and Kanter, 1964; Clark and Lasagna, 1965; Leonards and Levy, 1965), and the suggestion has been made that there may be a correlation between the blood levels obtained with various formulations (both enteric and non-coated) in the same individual (Truitt and Morgan, 1962; Levy and Hollister, 1964; Clark and Lasagna, 1965). This suggests significant differences in residence times of nondisintegrating solid dosage forms in the stomach (Blythe, Grass and MacDonell, 1959; Leonards and Levy, 1965). A more plausible suggestion is that the variability in observed blood levels are due to large individual differences in rates of salicylate elimination (Levy and Hollister, 1964a; Wood and Syarto, 1964; Bedford, Cummings and Martin, 1965; Levy, 1965; Paulus, Siegel, Mongan, Okun and Calabro, 1971). Evidence suggests that the overall rate of salicylic acid elimination can be described by a first order process only when the amount present in the body is less than 0.3 g (approximately 2.0 mmoles), (Bedford, Cummings and Martin, 1965; Levy, 1965a).

After a single one g dose of ASA, the mean half-life of salicylate has been reported to be six hours (Brodie, Burns and Weiner, 1959), while after very high doses, a value of nineteen hours has been obtained (Swintosky, 1956). These dose-dependent elimination kinetics are due to two capacity limited processes involved in SA metabolism. One saturable process is that of glycine conjugation to form salicyluric acid (Levy, 1965; Levy, 1965a; Bedford, Cummings and Martin, 1965; Cummings, Martin and Renton, 1966; Levy and Amsel, 1966; Amsel and Levy, 1970). The second rate-limiting process is that of glucuronide formation (Levy, Tsuchiva and Amsel, 1972; Tsuchiya and Levy, 1972). These two metabolites account for the majority of salicylate detoxification. Saturation of the processes leading to the formation of the major metabolites, results in an increase in the fraction of unconjugated salicylic acid excreted and the production of minor metabolites, such as gentisic acid, formed (Alpen, Mandel, Rodwell and Smith, 1951; Boreham and Martin, 1969).

III. Gastric Absorption Interactions

Salicylates have beem implicated in numerous drug-drug interactions and various explanations have been postulated for these interactions. Among them are: pharmacological potentiation, both additive and synergistic;

displacement from plasma or tissue proteins, enzymatic interference, either induction or inhibition of metabolizing enzyme synthesis or competition for limited enzyme action; interference with urinary excretion; and interference with drug absorption. The latter group of interactions was singled out for further investigation.

There are few known mechanisms regarding the interference of drugs and exogenous compounds on salicylate absorption. One proposed mechanism involving SA and caffeine is that these two compounds form a 1:1 soluble complex at pH 1.3, the result of which is decreased absorption from the rabbit stomach (Goto, Takamutsu, Shibao and Iguchi, 1968). This observation may help to explain previous data that the caffeine in ASA, caffeine and phenacetin mixtures inhibits the observed augmentative analgesic effect of the mixtures in mice (Grotto, Dikstein and Sulman, 1965).

Acetazolamide has been shown to increase the absorption of SA from rat intestinal sacs in an in vivo experiment (Schnell and Miya, 1970). The mechanism postulated is that the decrease in pH due to carbonic anhydrase inhibition decreases the amount of ionization of SA and renders it more readily absorbable. This hypothesis may be criticized on the basis of the known relative insensitivity of intestinal carbonic anhydrase to acetazolamide inhibition (Goodman and Gilman, 1970).

Certain cations affect the passive intestinal

absorption of drugs. Both ammonium and potassium ions were shown to decrease salicylate transfer from rat jejunum *in vitro* (Mayersohn and Gibaldi, 1970). No mechanism was proposed for the inhibition, although it was noted that the effect is dependent on the concentration of cation and is directly related to cation permeability.

There is some evidence to suggest an interaction between indomethacin and ASA. One study has proposed a decreased and delayed oral absorption of radioactive indomethacin when administered with ASA to arthritic patients (Jeremy and Towson, 1970). When administered intravenously to rats, decreased plasma levels of radioactivity were observed. Salicylic acid given intravenously or orally to rats from one to three hours after the administration of radioactive indomethacin resulted in decreased plasma radioactivity (Yesair, Remington, Callahan and Kensler, 1970). Concommitant urinary excretion of radioactivity decreased while bilary and fecal excretions increased. More recent evidence from human studies suggests the proposed interaction between ASA and indomethacin does not exist, and warns of the pitfalls of using non-specific isotope labeling methods (Champion, Paulus, Mongan, Okun, Pearson and Sarkissian, 1972).

Evidence regarding the effect of ascorbic acid on salicylate absorption is scant and conflicting. It has been reported (Denko, 1961) that ascorbic acid, p-aminobenzoic

acid and ASA combinations produce lower serum salicylate levels in arthritic patients that did ASA alone. The dose of ascorbic acid used (20 mg) was very low, and any proposed interaction would be minimized. Furthermore, these combination tablets were enteric-coated, and presumably passed through the stomach intact. Then rapid intestinal absorption would again minimize any proposed interaction.

More recently (Staudacher and Müller, 1969) it has been observed in human volunteers that combination tablets of 660 mg ASA plus 400 mg ascorbic acid produced significantly higher blood levels than did ASA alone. These observed differences were significant for up to two hours after oral administration, which suggests at least partial stomach involvement in the interaction. One possible explanation of this effect is that it may be due to formulation differences between the test and control dosage forms. This may result in large differences in tablet disintegration rates and consequent salicylate absorption. It was this reported interaction which was singled out for further investigation.

IV. Objectives of This Study

The objectives of this study were as follows:

a) To develop a method for the simultaneous estimation of ASA and SA from plasma samples as their TMS derivatives

using GLC,

b) To apply the method to an in vivo determination of bioavailability of various formulations of ASA, and

c) To employ the method to study the proposed interaction of ascorbic acid on ASA absorption. Any possible effect of ASA on ascorbic acid absorption was also to be noted.

METHODOLOGY

I. Instrumental

All salicylate analyses, with the exception of the combined gas liquid chromatography-mass spectrometry (GLC-MS) studies, were conducted on a Perkin-Elmer model F-11 gas chromatograph, equipped with dual flame ionization detectors. The signal from the detectors was fed into a Hewlett-Packard model 7127A strip chart recorder, with a 1 mv full scale response and a chart speed of 0.5 in $\min^{-1}$. Dual coiled glass columns, two metres in length and 4 mm internal diameter, containing 3% of OV-25 liquid phase coated onto 60/80 mesh Chromosorb G (regular) were used in the analyses. The operating temperatures were: injectors, 240°; oven, 160° (isothermal); and detectors, 240°.

The theory of gas chromatography has been adequately reviewed in several texts (*i.e.*, Heftmann, 1961; Szymanski, 1964; Chatten, 1969; and Littlewood, 1970), and will not be dealt with extensively here.

Column efficiency is a function of the linear carrier gas velocity, and to determine maximum efficiency, the flow rate of the carrier gas, helium, was varied whilst all other parameters were held constant (Figure 1). The number of theoretical plates, N, at each flow rate was calculated from the following formula (Heftmann, 1961):

$$N = 5.54 \frac{L^2}{(W1/2)^2}$$

where L is the distance from the point of injection to the

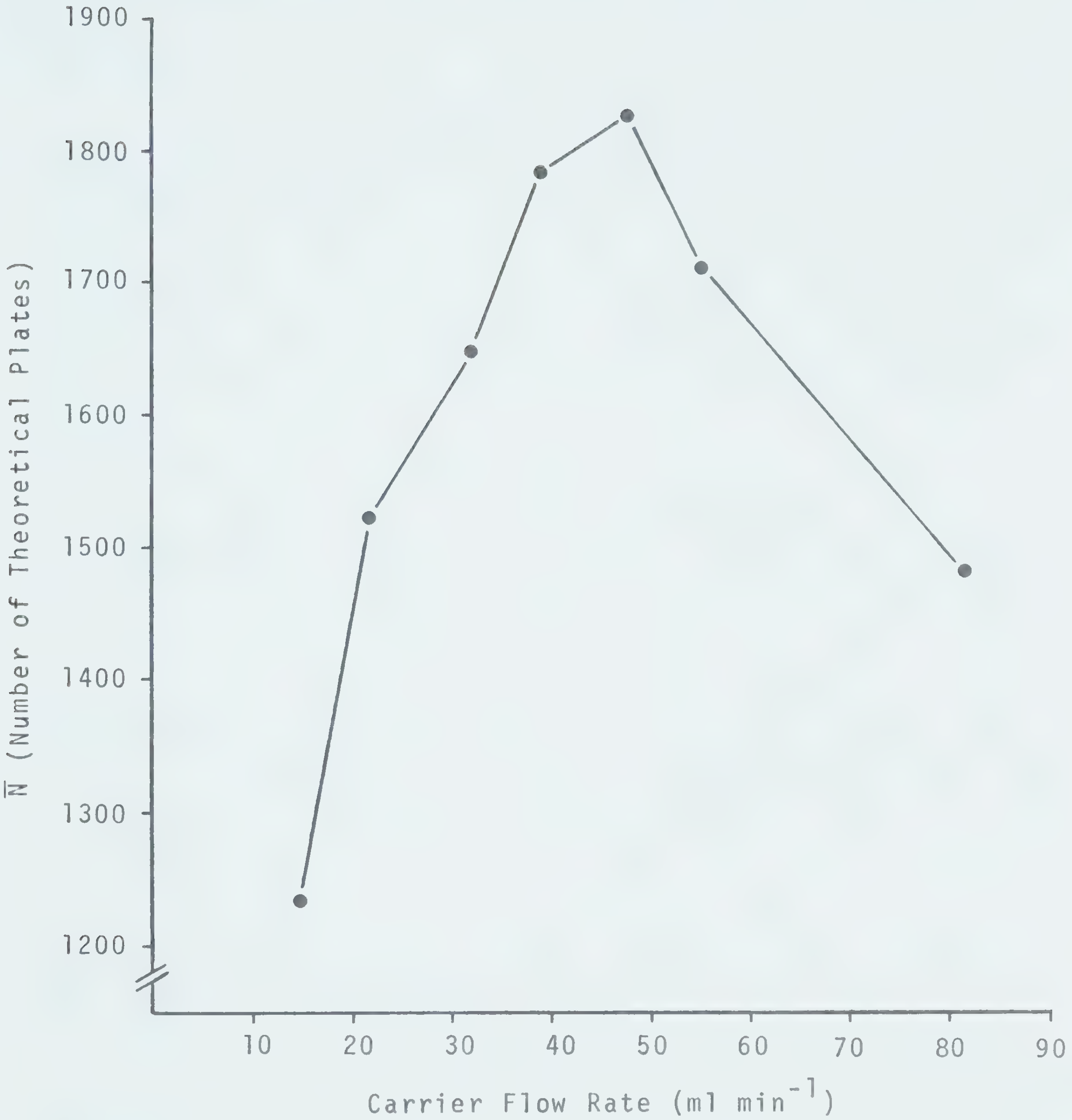


Figure 1: Plot of Mean Number of Theoretical Plates versus Carrier Gas Flow Rate for Columns of 3% OV-25 on Chromosorb G (regular) 60-80 mesh

(Each point represents the mean of three determinations)

center of the peak, and W1/2 is the peak width at half height.

The HETP, or height equivalent to one theoretical plate, may be calculated by dividing the mean number of theoretical plates at each carrier flow rate by the column length (Figure 2). According to the plate theory of separation, the larger the number of theoretical plates present, the greater will be the separation of individual component peaks.

Extrapolation from Figure 1 indicates that the maximum number of theoretical plates is approximately 1800, and this figure results at a linear gas velocity of 48 ml min^{-1}. In subsequent experiments a flow rate of 55 ml min^{-1} was employed, which corresponds to about 1700 plates. This figure is considerably less than a recommended value of 5000 plates for a 6 ft analytical column (Heftmann, 1961, p. 181). The columns used here produced adequate separation of the components within a satisfactory length of time in spite of the relatively small number of plates.

At 55 ml min^{-1}, the combination of air flow of approximately 600 ml min^{-1}, and of hydrogen flow of 40 ml min^{-1}, produced an optimal balance between maximum detector sensitivity and frequent flame extinction by solvent in the injected sample.

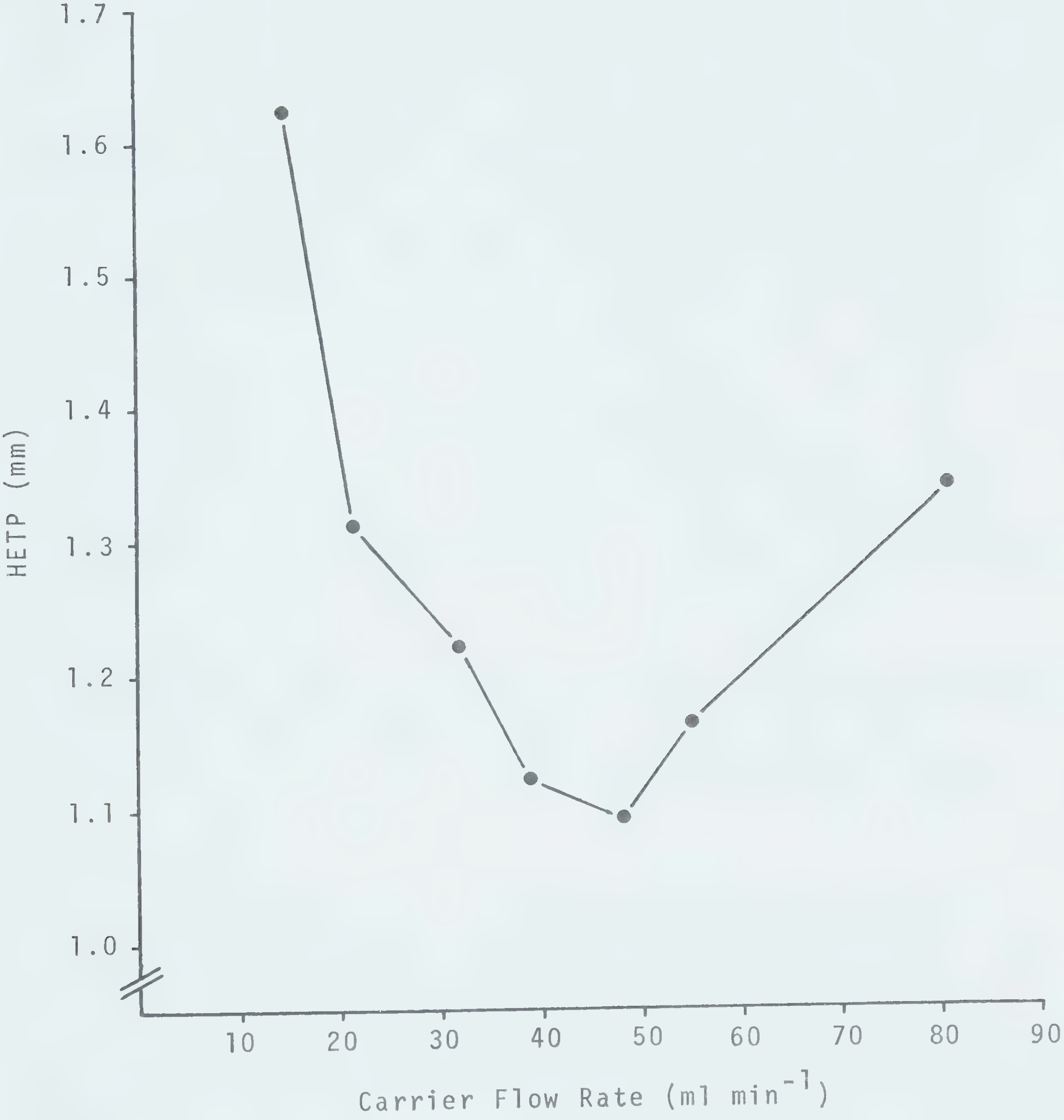


Figure 2: Plot of HETP versus Flow Rate for Columns of 3% OV-25 on Chromosorb G (regular) 60-80 mesh

(Each point represents the mean of three determinations)

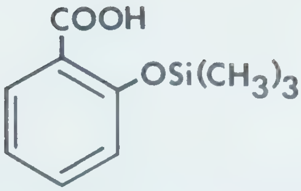
II. Silylation Studies

The structure of SA suggests that it should be possible to obtain up to three individual trimethylsilylated (or silylated) derivatives. Multiple GLC peaks have been reported previously from attempts to silylate SA (Rowland and Riegelman, 1967; Watson, Crescuolo and Matsui, 1971). In these preliminary experiments two peaks were obtained in variable ratio as silylation products of SA. Previous workers (Burkhard, 1957; Choby and Neuworth, 1966) had synthesized two of the three possible structures, namely 2-trimethylsiloxybenzoic acid (MSSA), and trimethylsilyl, 2-trimethylsiloxybenzoate (DSSA). No evidence was presented for the existence of the third possible derivative, 2-hydroxytrimethylsiloxybenzoate. This observation is in agreement with the general order of decreasing ease of silylation, which places phenols before carboxylic acids (Pierce, 1968).

In contrast, ASA can produce only one silylated derivative, trimethylsiloxyacetylsalicylate (ASA-TMS). The structure of the silylated derivatives, their molecular weights and chromatographic retention times are given in Figure 3.

a) GLC-MS Data

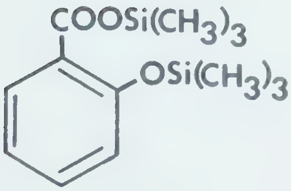
Mass spectral data, obtained from combined GLC-MS is summarized in the line diagrams of Figures 4 and 5. These low resolution mass spectral studies were conducted by



MSSA (2-trimethylsiloxybenzoic acid)

M.W. = 210

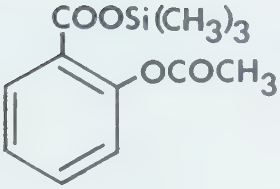
Retention Time = 2.1 min



DSSA (trimethylsilyl,2-trimethylsiloxybenzoate)

M.W. = 282

Retention Time = 4.9 min



ASA-TMS (trimethylsiloxyacetylsalicylate)

M.W. = 252

Rentention Time = 8.6 min

Figure 3: Structures of Silylated Salicylates

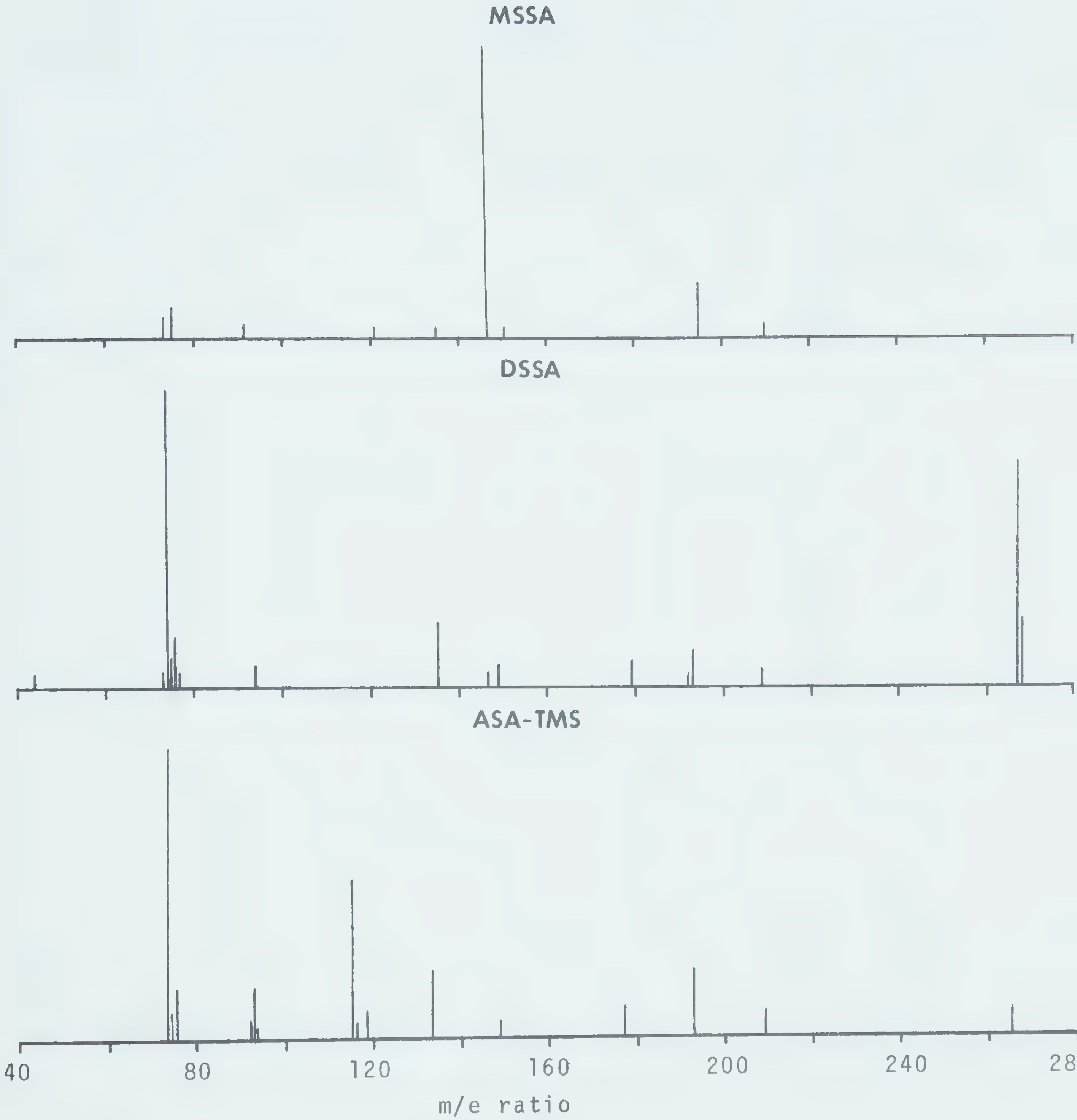


Figure 4: Mass Spectral Line Diagrams of Silylated Salicylates at 20 eV

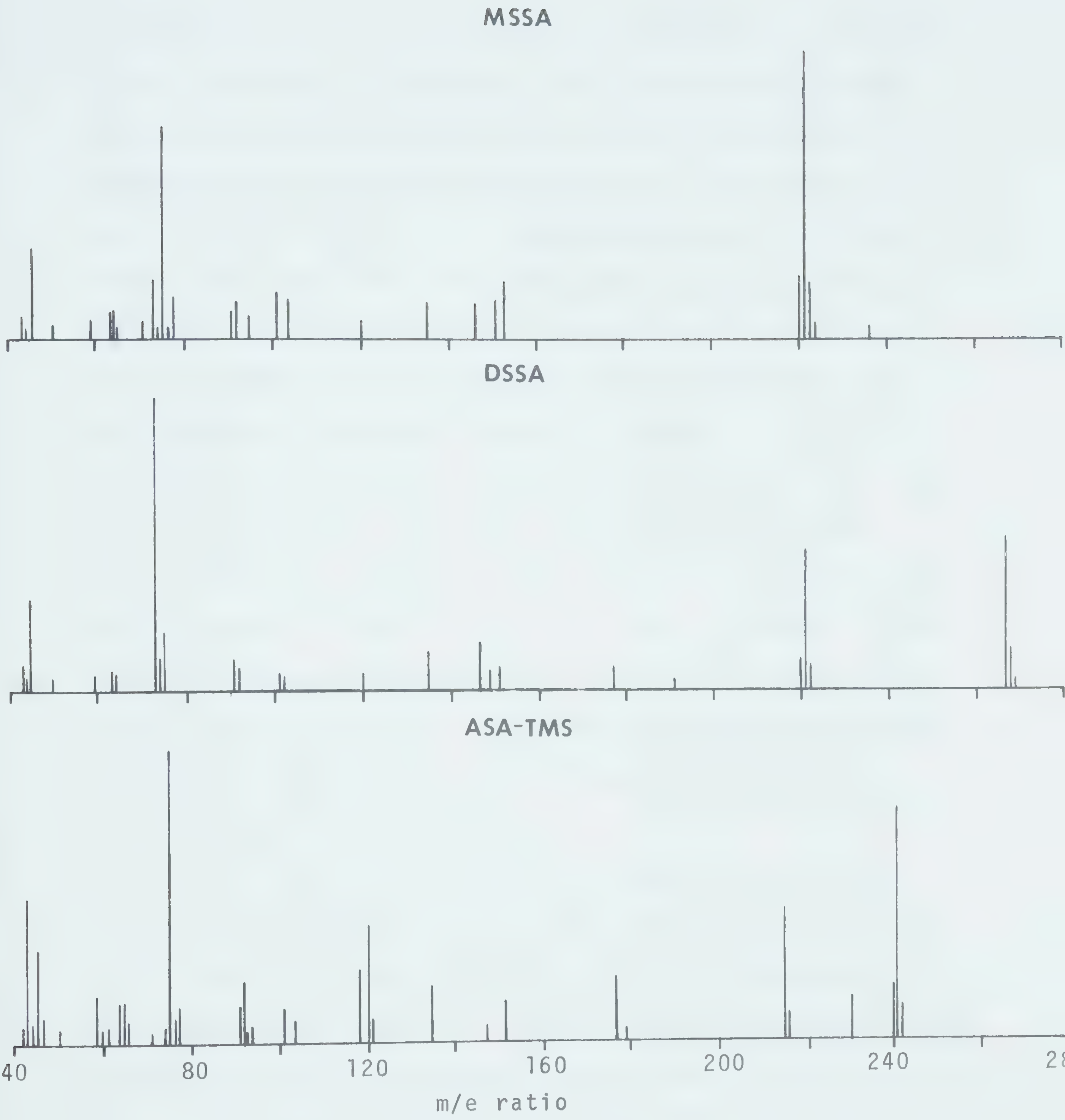


Figure 5: Mass Spectral Line Diagrams of Silylated Salicylates at 70 eV

Dr. A.M. Hogg and associates of the Department of Chemistry at the University of Alberta. The silylated salicylates were scanned at both 20 and 70 eV. A Varian 1200 gas chromatograph, coupled to an AEI-MS2 mass spectrometer was used in these studies. This chromatograph was a single column instrument, and the column packing and operating conditions were identical to those mentioned previously.

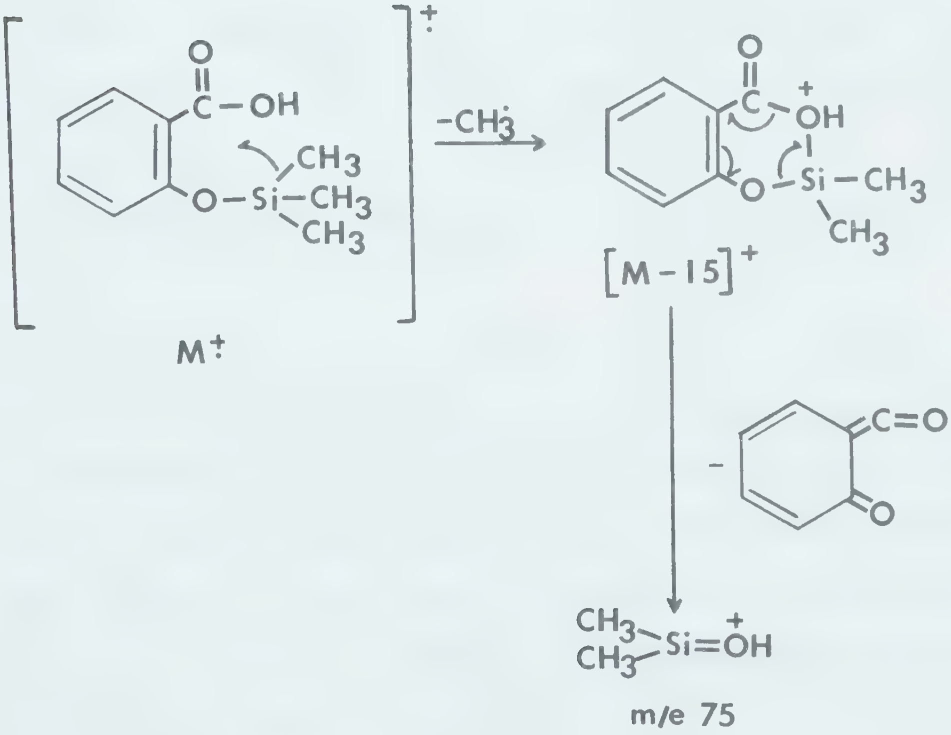
The mass spectrum of MSSA was consistent with the corresponding structure proposed in Figure 3. A 20 eV spectrum showed characteristic ions at m/e 210 (5.4%, $M^{+\cdot}$); 195 (17.3%, $M\text{-}15^+$); 151 (5.5%, $M\text{-}15\text{-}CO_2^{\ +}$); 75 [12.5%, $(CH_3)_2Si{=}\overset{+}{O}H$]; and 73 [8.7%, $(CH_3)_3Si^+$]. In addition, the 70 eV spectrum of MSSA had an ion at m/e 45 (30.8%), shown by accurate mass measurement to be $COOH^+$. The existence of the latter ion is taken as further evidence against the synthesis of the other possible isomer of MSSA, namely 2-hydroxytrimethylsiloxybenzoate.

Both the 20 eV and the 70 eV mass spectra of DSSA showed the following characteristic ions: m/e 267 (77.4%; $M\text{-}15^+$); 209 [5.5%, $M\text{-}Si(CH_3)_3^{\ +}$]; 193 [4.9%, $M\text{-}OSi(CH_3)_3^{\ +}$]; and 73 [100.0%, $(CH_3)_3Si^+$]. These ions are consistent with a previously published spectrum of the disilylated derivative of SA (Mamer, Crawhall and Tjoa, 1970) and support the proposed structure of DSSA in Figure 3.

The mass spectrum of ASA-TMS at 20 eV shows ions at m/e 193 [23.0%, $M\text{-}OSi(CH_3)_3^{\ +}$] and 73 [100.0%, $(CH_3)_3Si^+$].

In addition the corresponding 70 eV spectrum showed ions at m/e 210 (15.2%, $M-CH_2=C=O^{+\cdot}$); 195 (44.8%, $M-42-15^{+}$); and 135 [20.0%, $M-COOSi(CH_3)_3^{+}$]. The loss of $CH_2=C=O$ (42 mass units) is characteristic of acetates.

The mass spectral data obtained in these studies is conflicting and difficult to interpret. For example, the ions of m/e 220, 221, 222, 223 and 236 in the 70 eV scan of MSSA are obviously the result of ion formation in the mass spectrometer. The presence of an ion at m/e 75 for MSSA may be explained as follows:



Both scans of DSSA show fairly strong peaks at m/e 75. This ion could not be formed in a manner similar to that shown for MSSA because of the absence of the acid H atom.

The 70 eV spectrum of ASA-TMS shows the same spurious ions (m/e 220, 221, 222, 223, and 236) as found in the 70 eV MSSA trace and presumably arise in the same fashion. The absence of the M-42 ion in the 20 eV spectrum of ASA-TMS is also difficult to rationalize.

A typical chromatogram is depicted in Figure 6. This trace was obtained from a 1 ml sample of plasma from a subject one hour after the ingestion of 1.95 g of ASA in a buffered formulation. The first peak following the solvent peak is n-butyl benzoate (BB); the internal standard for peak 2, DSSA. The break in the trace between peaks 2 and 3 is due to a change of attenuation required to magnify the ASA-TMS peak which renders it easier to quantitate. Peak 3 is ethyl anisate (EA); the internal standard for peak 4, ASA-TMS.

b) Method of Quantification

The method of quantification chosen in these studies was one of internal standardization. Two such standards were used because of the large difference in plasma concentrations of SA and ASA. Internal standards were used for the following reasons:

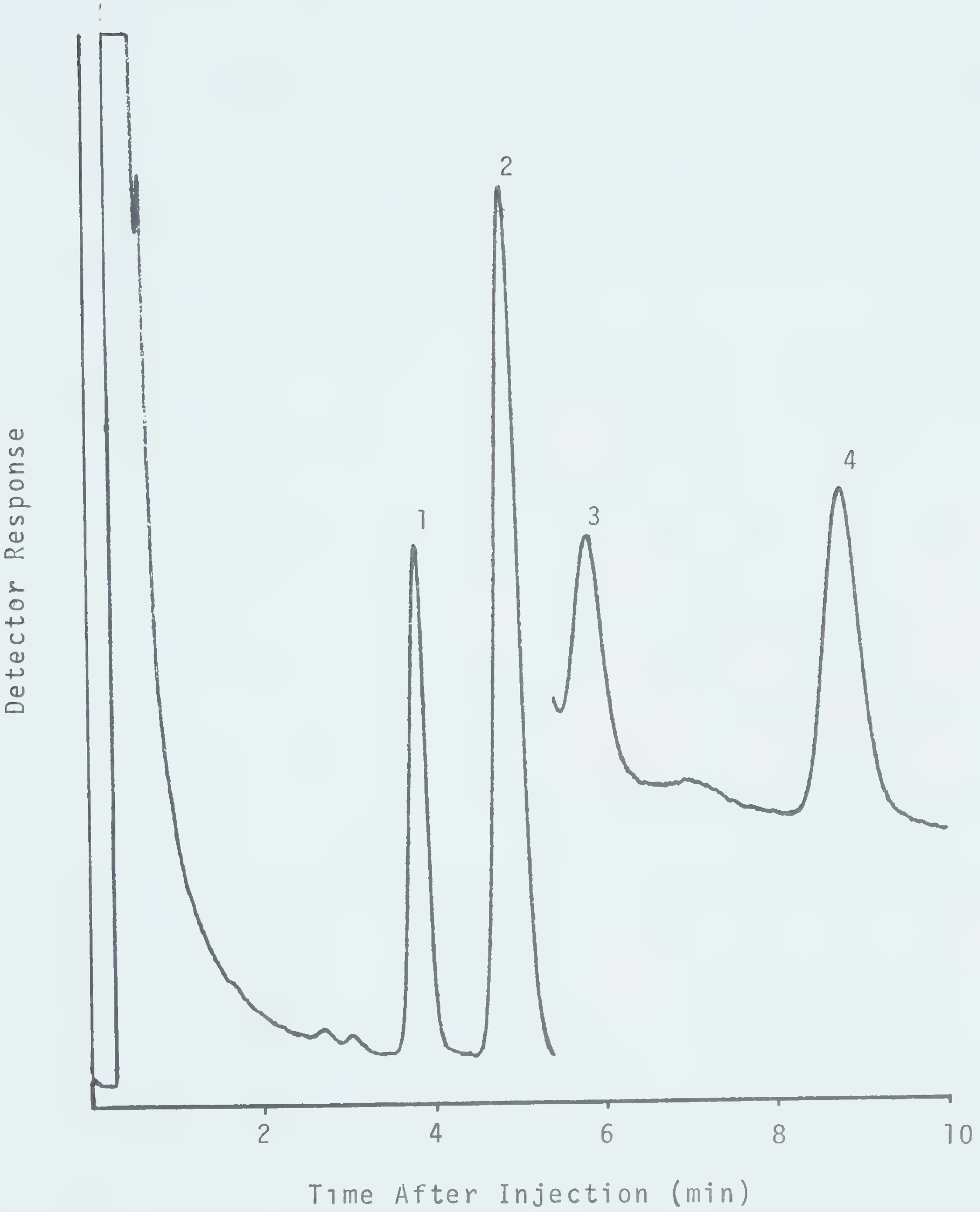


Figure 6: Typical Chromatogram

Key: 1 Butyl Benzoate
2 DSSA
3 Ethyl Anisate
4 ASA-TMS

1) It is difficult to inject exactly the same amount of sample mixture into the chromatograph each time. With internal standards, the ratio of a particular peak area to its internal standard remains unchanged, regardless of variations in sample size.
2) In case some of the sample solution is accidently lost during handling procedures, the peak area ratio of a particular peak to its internal standard will not change, although their absolute sizes will both be reduced.
3) Repeated injections of samples containing excess silylating reagents resulted in a gradual diminution of absolute detector response. Its relative sensitivity, in the presence of internal standards, remains unchanged.

Areas of the two salicylate peaks and their internal standards were determined by triangulation, and then the peak area ratios, DSSA/EA and ASA-TMS, were calculated. Calibration curves, constructed by varying the weights of salicylates with constant weights of internal standards, were used to determine the weight ratios of the unknowns involved. Because the weight of internal standard is kept constant, knowledge of the weight ratios permits calculation of the amounts of salicylates present.

Triangulation was selected as the means of peak quantification over other methods such as peak height measurement and peak weight determination. Peak area measurements are less subject to variation due to small fluctuations in

carrier flow rate than is peak height. Peak weight determinations are destructive and also more tedious than triangulation. The use of a planimeter or electronic integrating equipment gave no better results.

In order to obtain traces such as that shown in Figure 9, the following four factors were found to be of critical importance:

i) Time for reaction
ii) Silylation reagent used
iii) Solvent used
iv) Presence of moisture

c) Time for Reaction

Figure 7 shows an experiment in which the disilylation of SA was monitored over a 250 minute period. In order to cover the expected range of concentrations of SA in 1 ml of plasma, two concentrations (25 μg and 150 μg) of SA were studied. Similarly, Figure 8 shows the monosilylation of ASA (5 μg and 25 μg).

It is apparent from these two figures that the silylation reactions run to completion in approximately 50 minutes, as there are no appreciable increases in peak area ratios past that point. Furthermore, both DSSA and ASA-TMS, synthesized in this fashion are stable for a minimum of four hours after the addition of the reagent. Decomposition of the derivatives would be reflected in decreased peak area ratios. In all subsequent studies, 60 minutes was allowed

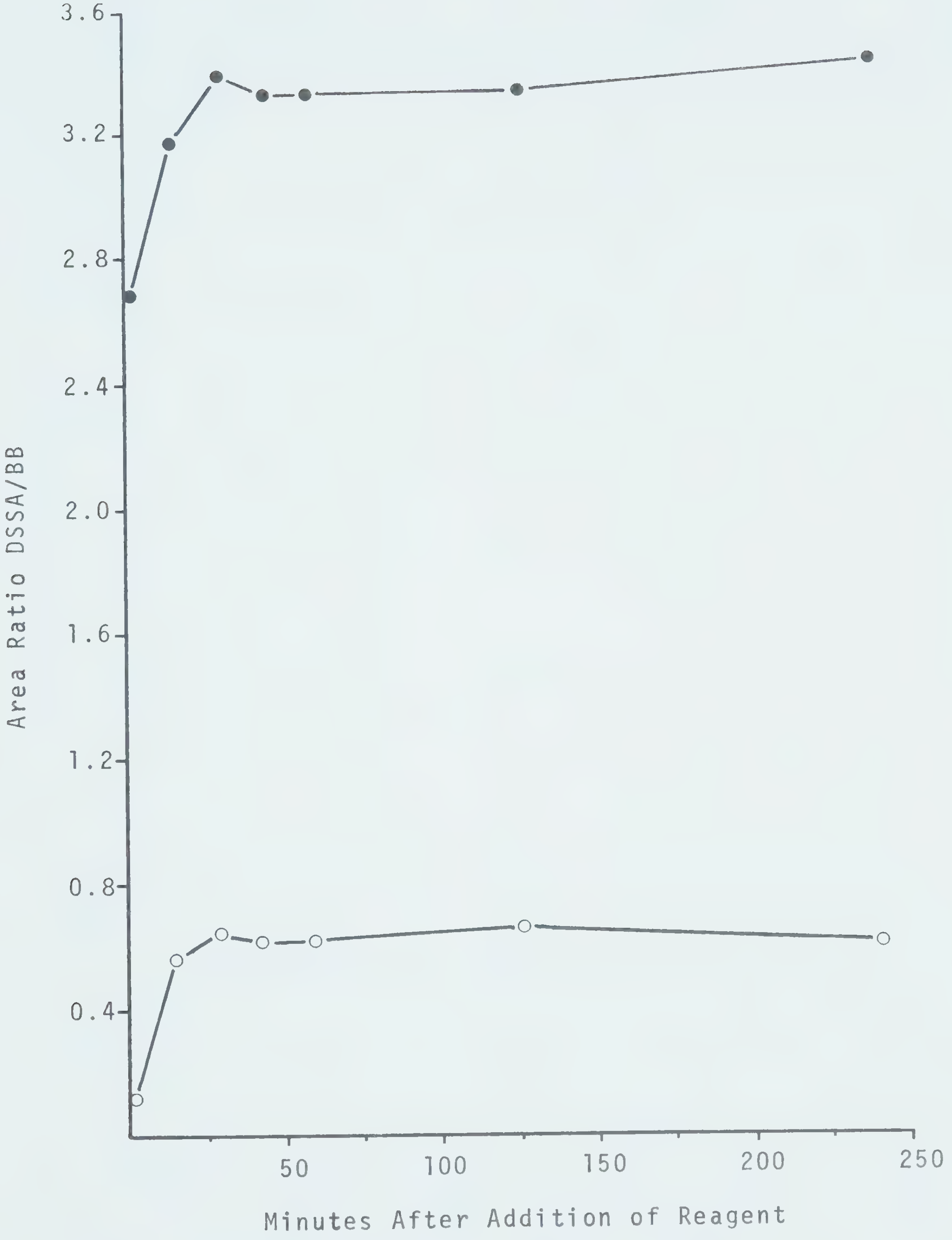


Figure 7: Time Required for Disilylation of Salicylic Acid

Key: ●——● 150 μg SA and 25 μg BB

○——○ 25 μg SA and 25 μg BB

(Each point represents the mean of three determinations)

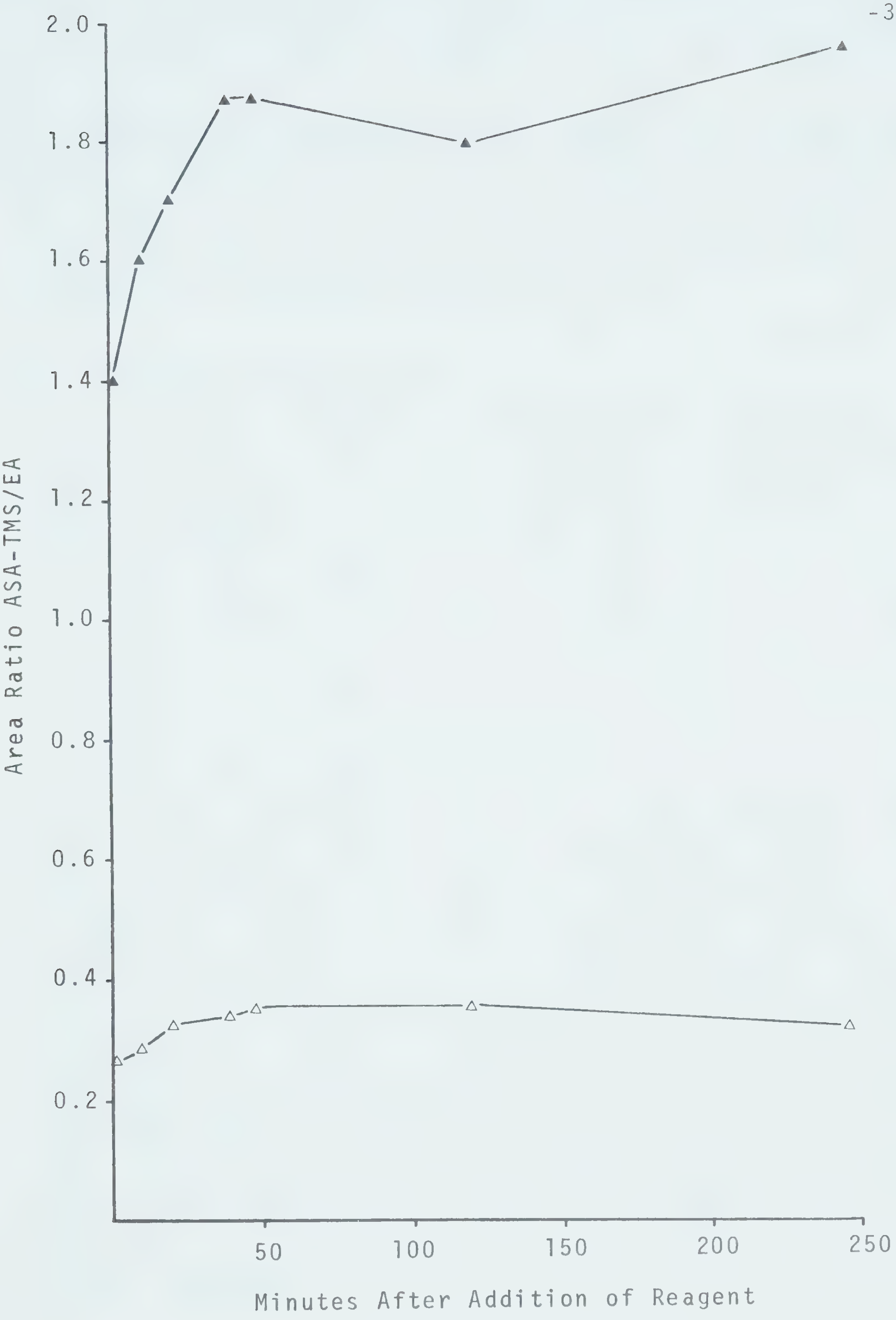


Figure 8: Time Required for Monosilylation of Acetylsalicylic Acid

Key: ▲——▲ 25 μg ASA and 0.5 μg EA

△——△ 5 μg ASA and 0.5 μg EA

(Each point represents the mean of three determinations)

to elapse between the addition of reagent and injection of a portion of the reaction mixture into the chromatograph. To minimize the time involved, successive samples were silylated at ten minute intervals.

d) Silylation Reagent Used

In order to determine the most useful silylating reagent for these studies, several common reagents and reagent mixtures were investigated:

a) 100 μl HMDS
b) 100 μl BSA
c) 100 μl TMCS
d) 50 μl HMDS and 50 μl TMCS
e) 80 μl HMDS and 20 μl TMCS

Fixed quantities of SA (150 μg) and of ASA (25 μg) were silylated with these reagents at room temperature (Figure 9). Neither bis (N,O-trimethylsilyl) acetamide (BSA), nor trimethylchlorosilane (TMCS), produced as large peak area ratios within 60 minutes as did hexamethyldisilazane (HMDS). Mixtures of HMDS and TMCS showed no advantage over HMDS alone, and the extent of silylation observed with these mixtures appeared to be dependent upon the amount of HMDS present. Consequently, HMDS was selected as the silylating reagent.

e) Solvent Used

Choice of solvents for silylation reations is somewhat

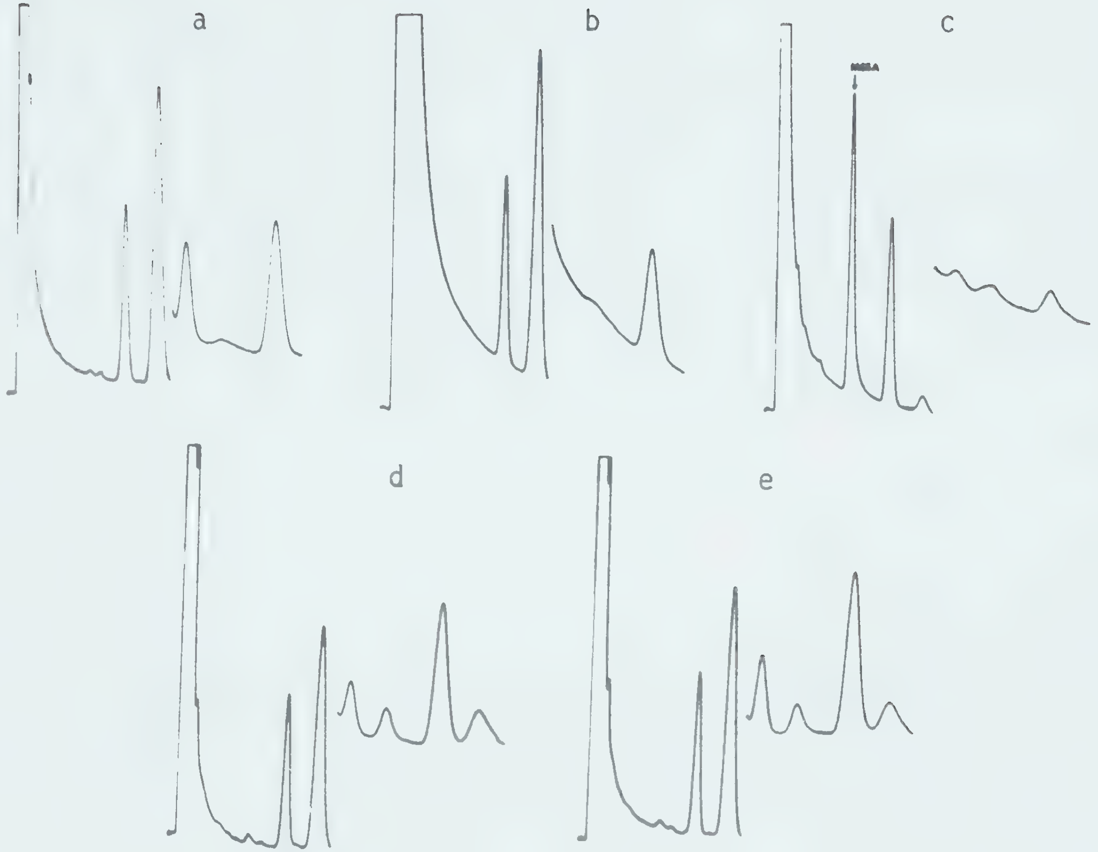


Figure 9: Effect of Reagent on Silylation

Key: a) 100 µl HMDS
b) 100 µl BSA
c) 100 µl TMCS
d) 50 µl HMDS and 50 µl TMCS
e) 80 µl HMDS and 20 µl TMCS

(Note that only HMDS and BSA produce complete disilylation of SA)

limited by the following:

i) The solvent must be inert to silylation. This requirement eliminated alcohols, phenols, thiols, primary and secondary amines, carboxylic acids, enolizable ketones, and other solvents which readily decompose into these compounds.

ii) The solvent must elute rapidly from the GLC column. This stipulation excluded most of the tertiary amines, even those of low molecular weight such as trimethylamine and triethylamine.

Using 100 μl of HMDS as the silylating reagent, and fixed quantities of SA (150 μg) and of ASA (25 μg), several common silylation solvents were studied (Figure 10). Inspection of Figure 10 reveals the presence of MSSA in the majority of the traces in 60 minutes. The presence of an MSSA peak indicated less than quantitative synthesis of DSSA. Therefore, the following solvents were eliminated: dimethylformamide, dioxane, chloroform, n-hexane, carbon disulfide and diethyl ether. This left two solvents, acetone and pyridine, both of which catalyze the disilylation of SA and the monosilylation of ASA to an equal extent. Pyridine, however, has a somewhat longer solvent tail than does acetone. This tail tends to overlap the salicylate peaks. For these reasons, acetone was selected as the solvent.

The size of the MSSA peak in several of the traces using nonpolar solvents suggested the possibility of

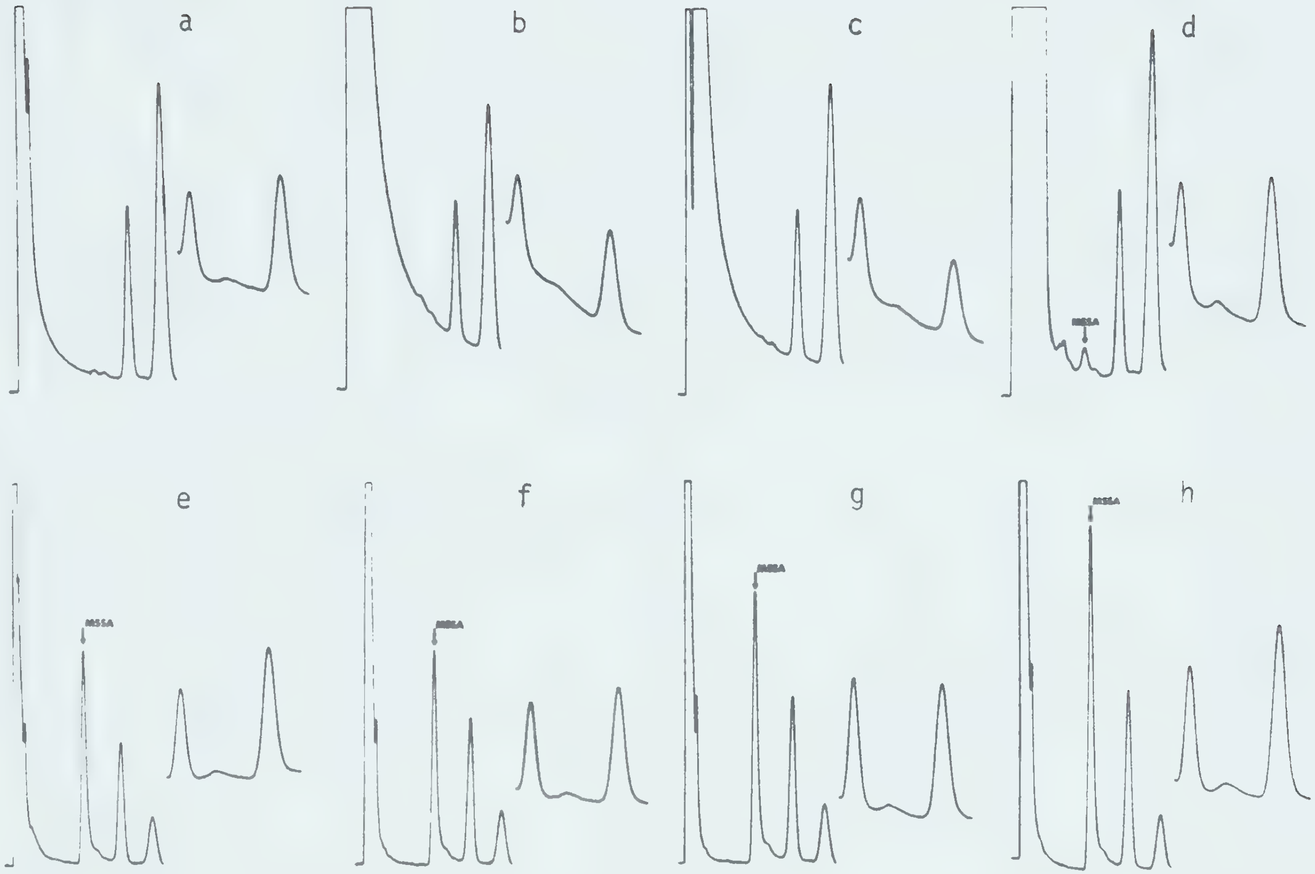


Figure 10: Effect of Solvent on Silylation

Key: a) 100 µl acetone
b) 100 µl pyridine
c) 100 µl dimethylformamide
d) 100 µl dioxane
e) 100 µl chloroform
f) 100 µl n-hexane
g) 100 µl carbon disulfide
h) 100 µl diethyl ether

(Note that only acetone and pyridine catalyze complete disilylation of SA)

quantitative MSSA synthesis. This alternative was investigated using carbon disulfide, ether and chloroform. In all instances, however, some DSSA was formed, usually in variable quantity, and linear calibration curves could not be obtained. On this basis it was decided to synthesize DSSA quantitatively.

f) Presence of Moisture

The fourth critical factor in these silylation reactions was moisture. The effect of moisture on the silylation of 150 μg SA and 25 μg ASA with 100 μl HMDS in 100 μl acetone, was investigated. It is shown in Figure 11 that the silylations are affected by the presence of moisture. The system appears to tolerate approximately 0.5% of water. ASA is somewhat more sensitive to moisture than is SA. Water in the reaction mixture is relatively slow to elute off the column, and produces a long solvent tail.

Preliminary experiments suggested that 25 μl of HMDS is sufficient to silylate a mixture of 150 μg of SA and 25 μg ASA in 100 μl acetone. On this basis, 100 μl of HMDS is enough to silylate approximately 600 μg of SA plus 100 μg of ASA. The maximum amount of SA (which is usually in vast excess of the amount of ASA) found in plasma samples in our studies, was never in excess of 200 μg ml^{-1}. This large excess of reagent compensates for any reagent lost in reactions with possible extraneous material.

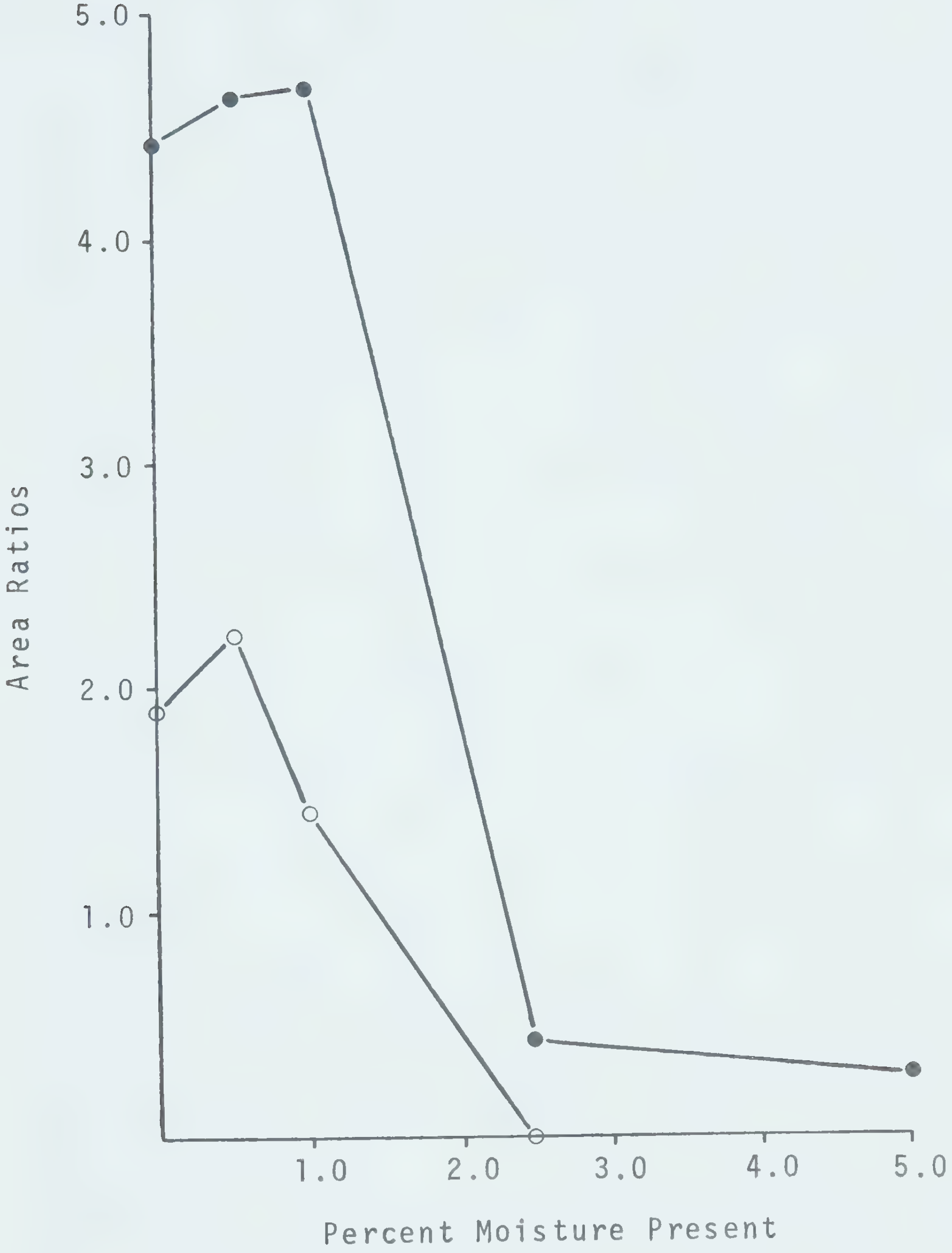


Figure 11: Effect of Moisture on Silylation

Key: ●——● 150 g SA and 25 μg BB

○——○ 25 g ASA and 0.5 μg EA

[Increased quantities of water resulted in decreased peak area ratios, suggesting increased breakdown of silylated derivatives, silylating reagent or both. (Each point represents the mean of three determinations)]

III. Plasma Extraction Studies

a) Acidifying Agent

Unacidified 1 ml plasma samples containing known quantities of SA and ASA yield approximately 4.0% of the SA and 6.0% of the ASA present when extracted into a suitable organic solvent. These extremely low recoveries clearly indicated the need to lower the pH of the plasma to facilitate partitioning into the organic phase. In order to improve recovery, numerous acidifying agents were investigated. Hydrochloric acid (0.5-6.0 N) produced considerable hydrolysis of added ASA. Trichloroacetic acid was found to extract into the organic solvent and hence neither of these two agents were considered suitable. Potassium bisulfate solutions have been employed successfully (Rowland and Riegelman, 1967a) and several concentrations were investigated here. The optimal concentration of $KHSO_4$ in terms of recovery of added salicylates was 10%. This concentration of $KHSO_4$ produced comparatively little ASA hydrolysis during extraction even though the pH of the acidified plasma was approximately 1.0. In subsequent experiments 1 ml plasma samples were acidified with 1 ml of 10% $KHSO_4$ solution. At this pH, the theoretical ratios of unionized : ionized forms of the salicylates is 100 : 1 for SA (pKa 3.0) and 300 : 1 for ASA (pKa 3.5).

b) Extraction Solvent

Several solvents were screened in an attempt to

obtain quantitative recovery of added salicylates. A single extraction procedure was selected in order to minimize handling and to reduce the time required for analysis. The solvents investigated were diethyl ether, methylene chloride, ethylene dichloride and chloroform. The highest and most consistent recoveries of salicylates added to blank plasma were obtained with chloroform and ethylene dichloride. Chloroform was chosen over ethylene dichloride on the basis of its higher volatility. For example, 4.0 ml of chloroform may be removed by flash evaporation at 20° in approximately seven minutes, while the same volume of ethylene dichloride requires nearly 15 minutes. The time difference becomes appreciable when large numbers of samples are involved. The extraction solvent must be removed completely in order to facilitate complete silylation with HMDS-acetone. Although ether is relatively easy to remove, it is difficult to handle due to its volatility. Ether also dissolves appreciable water when mixed with aqueous samples. Chloroform offered the best compromise between volatility and extraction of water.

Using chloroform as the solvent, the optimal extraction time was determined. A reciprocating mechanical shaking apparatus was used in these experiments. It was observed that ten minutes of shaking produced as high recoveries of added salicylates as did 40 minutes. In subsequent studies, all samples were extracted for 15 minutes at room temperature on the same shaking device.

c) Percent Recovery

To determine whether the percent extraction of SA and ASA remained constant over a wide range of concentrations, several combinations of SA and ASA were investigated (Table I). Ten replicates were used at each combination of salicylate concentrations. The salicylates were added in 100 µl ether to 1 ml plasma samples, and the ether was gently removed under a stream of nitrogen gas. The plasma samples were then acidified with 1 ml of 10% $KHSO_4$ solution. Five ml of chloroform were added and the samples extracted for 15 minutes, in 15 ml glass-stoppered centrifuge tubes. The samples were then centrifuged to separate the layers, and 4.0 ml aliquots of the chloroform layers were recovered

Salicylates Added		Percent Recovery	
µg SA plus µg ASA		SA	ASA
150	5	90.6 ± 9.2	80.1 ± 16.3
150	25	96.4 ± 4.5	75.2 ± 9.8
25	25	92.7 ± 10.3	66.5 ± 15.0
25	5	82.7 ± 15.8	63.5 ± 37.2

Table I

Percent Extraction of Various Combinations of SA and ASA

uniformly. The internal standards were added to the extracts and the samples evaporated to dryness on a flash evaporator at 20°±2°. Flash evaporation was selected over evaporation under a stream of dry nitrogen gas because the latter method resulted in considerable losses of internal standards. No such losses were observed during flash evaporation at 20°C.

The evaporated samples were then reconstituted with 100 µl acetone and silylated with 100 µl HMDS and left at room temperature for 60 minutes before analysis. Non-extracted controls were used to determine the percent extraction.

Inspection of Table I suggests that within the range of concentrations studied, the percent extraction of both salicylates is independent of concentration. The mean percent extraction with this single extraction procedure was found to be 90.6±5.8 for SA and 71.3±7.6 for ASA (standard deviations).

d) Standard Calibration Curves

Calibration curves were constructed for both SA and ASA in the following manner. Known and varying amounts of salicylates were added to separate 1 ml portions of plasma. The salicylates were extracted and analyzed as outlined. The observed area ratios of DSSA/BB and ASA-TMS/EA were plotted against the known weight ratios. Figure 12 represents such a curve for SA. Each point represents eight determinations, and the mean and standard deviation about the mean are included. A computer drawn regression line

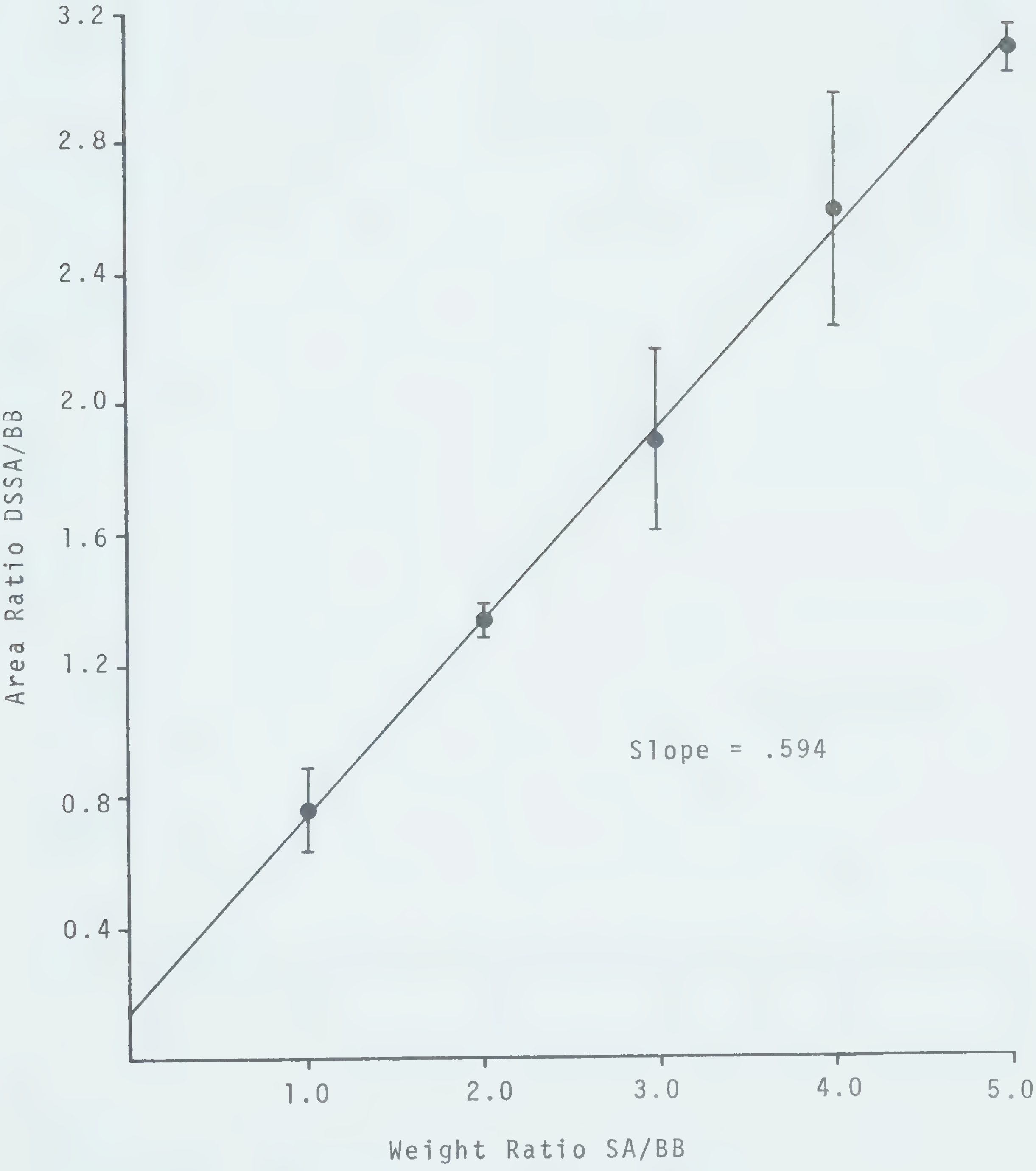


Figure 12: Extraction-Calibration Curve of SA versus BB

(Each point represents the mean of eight determinations with standard deviations)

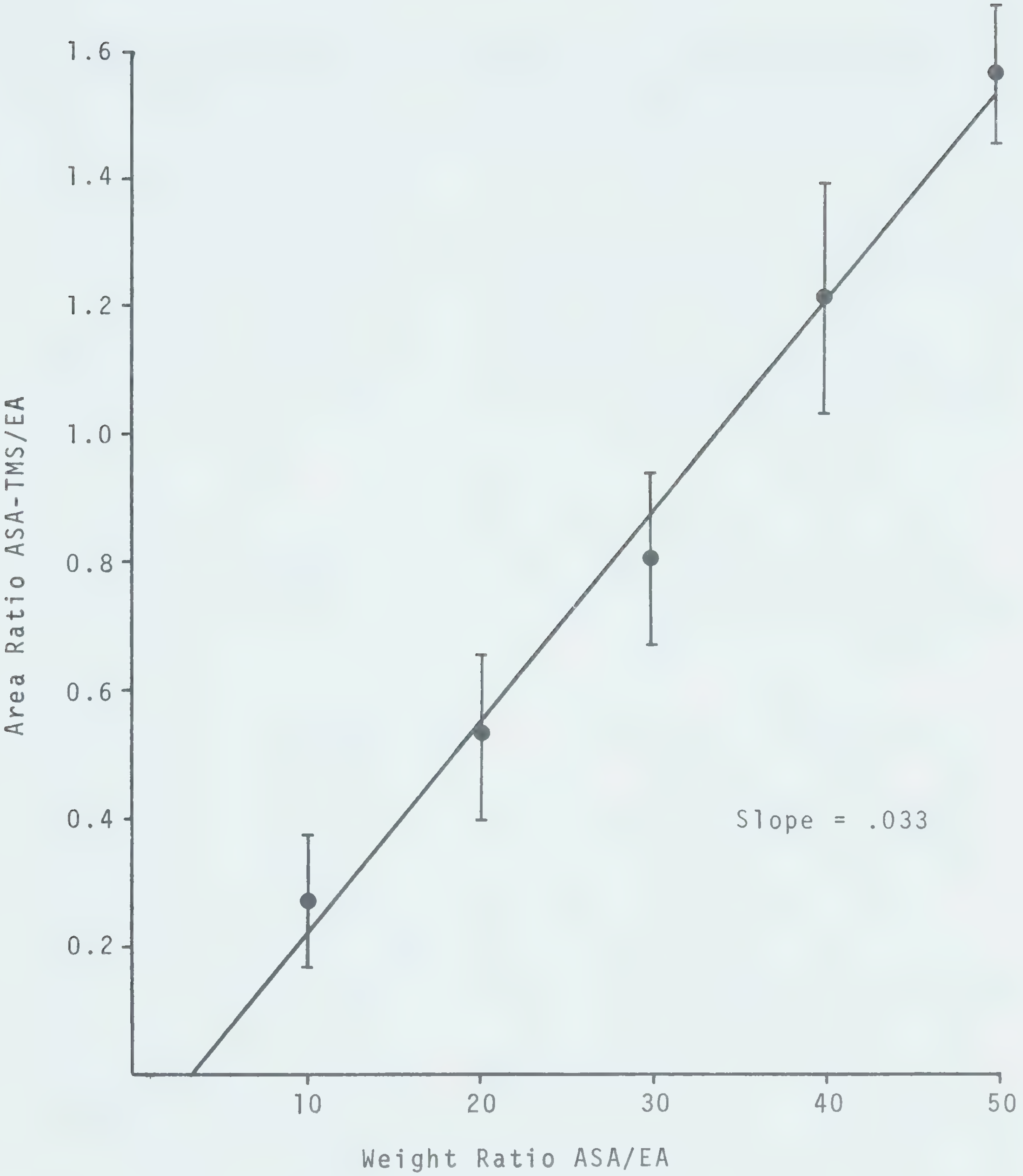


Figure 13: Extraction-Calibration Curve of ASA versus EA

(Each point represents the mean of eight determinations with standard deviations)

was employed to obtain the best fit to the experimental data. Figure 13 is the corresponding ASA curve.

e) Fluoride and Cooling

It had previously been demonstrated that the presence of fluoride in whole blood samples and cooling of the samples after drawing the blood resulted in considerable reduction of hydrolysis of ASA in the samples (Rowland and Riegelman, 1967a). The effects of fluoride and cooling were investigated as follows. Whole blood samples (7 ml) were drawn from volunteers into glass tubes containing 50 units of heparin, free of preservatives. Common preservatives such as benzyl alcohol may be extracted, silylated and subsequently chromatographed. The blood was immediately centrifuged, the plasma was pipetted off and mixed at room temperature. Four 10 ml aliquots of the pooled plasma were withdrawn. Two of the aliquots were warmed to 37° on a water bath, and the other two were cooled to 1° on ice. Then 50 μl of KF solution (25 mg KF) were added to one 'warm' and one 'cooled' aliquot. Subsequently, 25 μg ASA/ml of plasma was added to each of the four aliquots. The ether was removed, the samples mixed and frozen immediately on dry ice. The total elapsed time between the addition of the ASA and freezing of the samples was approximately 15 minutes. For the analyses, six 1 ml portions of each aliquot were assayed for SA. The results, summarized in Table II, suggest that both the presence of fluoride and the

Conditions KF	 0°	μg SA Present	μg ASA Hydrolyzed	% of ASA Hydrolyzed
-	-	6.07	7.92	31.7
-	+	3.35	4.37	17.5
+	-	3.62	4.72	18.9
+	+	2.46	3.21	12.8

Table II

Effects of Fluoride and Cooling on ASA Hydrolysis
(Note that a combination of fluoride and cooling resulted in least hydrolysis of ASA during the ten minutes of extraction)

cooling of the samples reduces the magnitude of hydrolysis, but the combination of fluoride plus cooling is best. In subsequent experiments whole blood samples were drawn into heparinized tubes containing fluoride. The samples were immediately cooled to approximately 1° on ice, and centrifuged. Plasma was then pipetted off, rapidly frozen on dry ice, and stored there until required for analysis.

f) Storage of Samples

Previous experimenters had observed that plasma samples containing ASA may be stored on dry ice for periods as long as 30 days without any appreciable decomposition to SA (Rowland and Riegelman, 1967a). In this experiment, 35 ml of pooled, heparinized plasma containing fluoride were used. The plasma was cooled to 1°, and 25 μg ASA/ml of

plasma were added. The plasma was well mixed, divided into four equal portions, rapidly frozen, and stored on dry ice. Individual portions were thawed and analyzed for SA after various storage periods (Figure 14). Each point on the curve represents seven determinations, and the mean and standard deviation about the mean are included. A computer drawn regression line was employed to determine the best fit to the experimental data.

The Y intercept at zero days storage in Figure 14 suggests that approximately 8.5% of the 25 µg ASA is hydrolyzed during the extraction procedure and subsequent handling (i.e., 1.62 µg of SA is produced by the decomposition of 2.12 µg of ASA). This figure is somewhat lower than that obtained in the fluoride and cooling experiment. The storage results are expressed in terms of SA production over time. The half-life of SA formation, and consequently of ASA decomposition, is 23.5 days, and the corresponding rate constant is .02949 day^{-1}. This rate is much lower than a quoted rate of 0.09372 day^{-1} at 17.2° and at pH 7.4 (Gore, Naik, Kildsig, Peck, Smolen and Banker, 1968).

The results of this experiment suggest that the rate of ASA decomposition in plasma is appreciable even when stored on dry ice at -79°. Ideally then, plasma samples containing ASA should be assayed as soon as possible after drawing them to avoid the corrections for ASA decomposition.

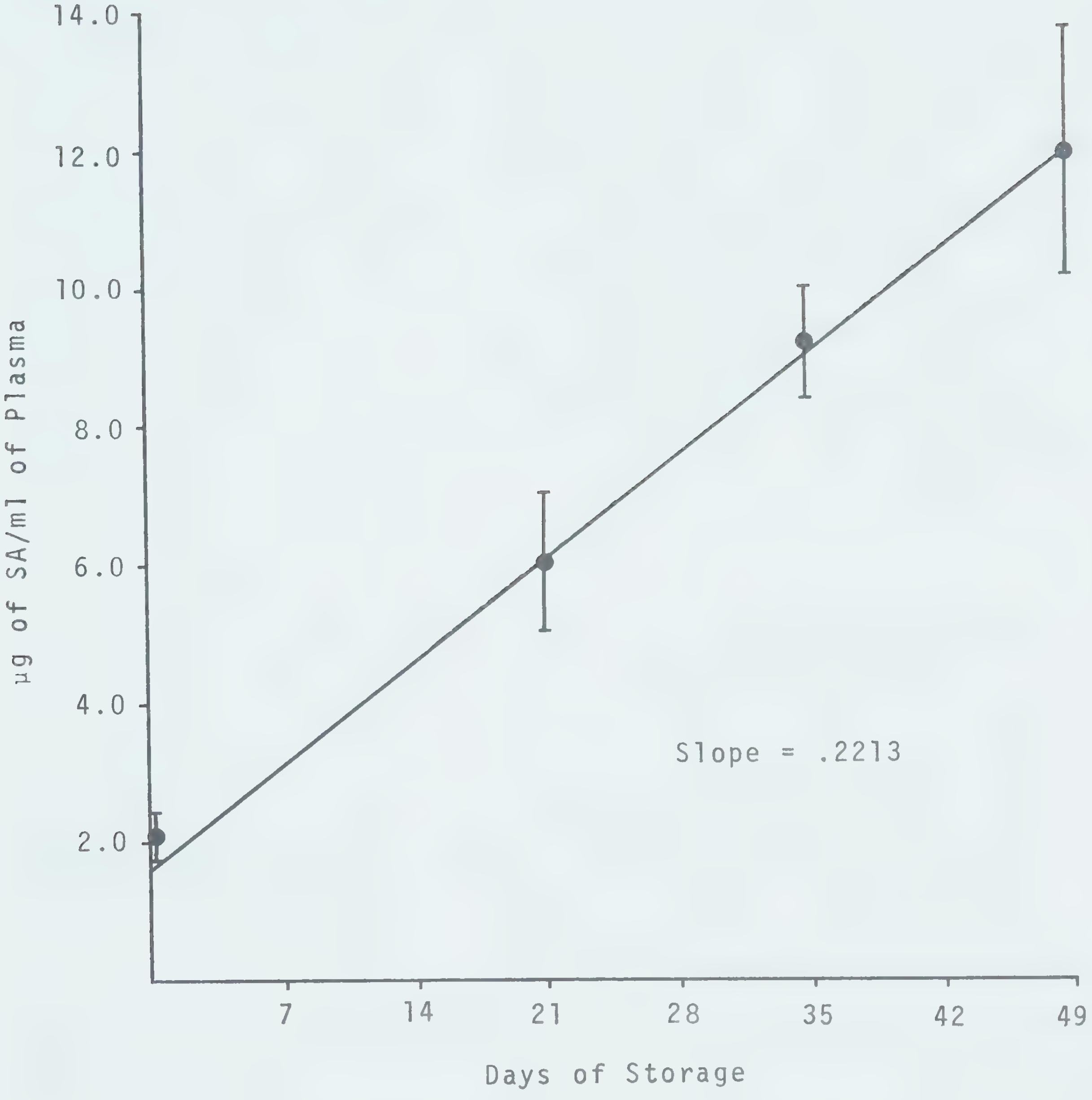


Figure 14: Effects of Storage on Decomposition of ASA

(Each point on the curve represents seven determinations with standard deviations)

IV. Comparison With Another Method

An experiment was set up to determine the comparative reproducibility of this GLC method of analysis with an established method. It was desirable that the established method be able to estimate both ASA and SA. Many of the fluorometric, ultra-violet and colorimetric techniques mentioned have this capacity. The method selected (Cotty, Zurzola, Beezley and Rodgers, 1965), is a colorimetric ferric ion complexation assay, specific for free SA (FSA). To estimate SA, aliquots of the same sample are hydrolyzed and assayed for total salicylic acid (TSA). The difference in absorbance of the two aliquots at 540 mμ is assumed to be due to ASA.

In this comparison, three lots of pooled heparinized human plasma were used. Various amounts of salicylates were added to each lot of plasma, and the samples were then rapidly frozen on dry ice. Portions of each lot were then thawed and analyzed by each of the two methods. Standards containing known similar quantities of salicylates as the test plasma were used to quantitate the results in each method. Each figure (Table III) represents the mean of four determinations.

The results in Table III suggest that the GLC method developed here is somewhat more precise for ASA and SA determination jointly but less precise for ASA or SA estimation alone than is the colorimetric assay.

Approximate Concentration (μg ml^{-1})	Results of Assays (μg ml^{-1})	
	GLC Method	Colorimetric Method
25 ASA	21.3 ± 2.9	25.0 ± 1.9
25 ASA plus 100 SA	20.9 ± 1.7 83.9 ± 11.3	4.7 ± 16.1 88.0 ± 17.8
100 SA	90.9 ± 7.5	85.6 ± 4.5

Table III

Comparison of GLC Method with Colorimetric Assay

(Each figure represents mean of four determinations plus or minus standard deviation)

V. Outline of Methodology

The procedure developed here may be briefly summarized as follows. Whole blood samples (7 ml) were drawn into evacuated glass tubes containing 50 units of heparin and 25 mg of KF in 100 μl of water. The contribution of this volume to the total is approximately 1.5%, and was ignored throughout.

The tubes containing the blood were immediately placed into an ice bath and cooled to approximately 2°. The plasma was then separated by centrifugation, frozen quickly on dry ice and stored there until required for assay. In some cases this was up to a year.

For the analysis, the plasma was slowly thawed at room temperature and duplicate 1 ml portions were pipetted into separate 15 ml glass stoppered centrifuge tubes. One ml of 10% $KHSO_4$ solution and 5 ml of chloroform were added to each tube. They were then stoppered and agitated lengthwise on a reciprocating mechanical shaker for 15 minutes. The tubes were then centrifuged to separate the phases and 4.0 ml of chloroform solution was removed from each tube. The internal standards, BB (25.0 μg) and EA (0.5 μg) were then added to each 4 ml aliquot of chloroform solution. These solutions were then carefully evaporated on a flash evaporator at 20° to dryness.

The contents of each tube were then reconstituted with 100 μl of acetone and then silylated with 100 μl HMDS

at room temperature for 50 minutes.

From 1 to 3 µl of the reaction mixture was then chromatographed isothermally at 160° on a column of 3% OV-25 on Chromosorb G (regular) 60/80 mesh.

The areas of the four resultant peaks (BB, DSSA, EA and ASA-TMS) were then calculated by triangulation, i.e. peak height multiplied by peak width at half height. The area ratios of DSSA/BB and ASA-TMS/EA were determined, and by reference to previously determined standard extraction calibration curves, the amount of SA and ASA in each 1 ml plasma sample was extrapolated.

It should be emphasized that a technique of positive controls was used throughout this work, both in the development of the methodology and during the assay of unknowns after the procedure had been established. Briefly, this involved the addition of known quantities of salicylates (usually 100 µg SA and 5 µg ASA) in ether to 1 ml samples of blank pooled plasma. The ether was allowed to evaporate, the standards mixed and then assayed along with and in the same manner as the samples of unknown concentrations. This provided a check on the mechanics of the procedure as well as on the reliability of the calibration curves.

Among the obvious criticisms of the technique are the following:

a) Because the procedure recovered only 4 of the original 5 ml of chloroform, some samples of low concentration

may erroneously be assayed to be blank. Quantitative recovery of the chloroform was not possible due to a variable degree of emulsification at the interface between the two phases. Even when corrected for the volume difference the single extraction procedure gave less than a quantitative recovery (Table I). The single extraction was a compromise between quantitative recovery and overall analysis time with its implications on the suitability of the method for routine use.

b) The method assumed that the recovery of salicylates from spiked plasma was identical to that of recovery of plasma salicylates following oral absorption. A further assumption was that there was no intersubject variation in the strength of protein binding of salicylates.

c) The control of hydrolysis of ASA between withdrawal of the blood and assay was less than ideal. When drawing blood samples from a number of patients it is inconvenient to centrifuge each sample and freeze it immediately. Rather, in this method a sample was obtained from each patient and then all four were centrifuged and frozen. It is reasonable to assume that the longer the interval between withdrawal of blood and freezing of the plasma, the greater was the ASA hydrolysis.

Storage on dry ice did not prevent the hydrolysis of ASA (Figure 14). During the analytical procedure itself

up to 8.5% of the ASA present may be hydrolyzed.

d) The method is not applicable to plasma containing hemolyzed blood cells. It was found that when hemolysis had occurred, many interfering components were extracted and chromatographed. Care must be taken to ensure the use of a needle of sufficient size as to avoid hemolysis.

e) The method is somewhat tedious. For example, one analyst may assay 20 duplicate plasma samples plus controls in 10 hours. Fortunately, the analysis may be stopped after the flash evaporation (before silylation) if necessary.

APPLICATIONS OF METHODOLOGY

I. Human Bioavailability Trial

a) Design of Trial

Subsequent to the development of the methodology for SA and ASA determination in plasma the following study was undertaken. The bioavailabilities of various brands of ASA, particularly enteric-coated varieties, were investigated, with the objective of assisting the selection of a particular formulation for routine administration to arthritic in-patients at the University of Alberta Hospital. Several brands of these tablets were available in Canada, but there was a paucity of information regarding their bioavailabilities and extremely little literature available concerning their comparative bioavailabilities. With these limitations in mind it was decided to conduct a trial comparing the *in vivo* bioavailabilities of the following formulations:

a)	Aspirin	(Bayer L 2095, 5 gr)
b)	Novasen	(Novapharm L 560, 5 gr)
c)	Entrophen	(Frosst L 011271, 320 mg)
d)	Bufferin	(Bristol-Myers L 567, 5 gr)
e)	Ecotrin	(Smith, Kline and French B 8095, 320 mg)

Volunteer arthritic in-patients of both sexes were included in the trial. Admission of patients was contingent on an adequate renal function as determined by creatinine clearance and blood urea nitrogen (BUN) tests, and on the absence of other complicating illnesses (i.e, peptic ulcer and gastric surgery). Other medication which could interfere

with salicylate absorption, plasma binding, metabolism or excretion were not permitted. This criterior eliminated such drugs as antacids, intestinal adsorbants, surfactants, diuretics, caffeine, ascorbic acid, phenylbutazone and coumarin anticoagulants.

Prior to admission into the trial, all forms of salicylate were withdrawn from the patients for a period of 72 hours to permit complete elimination of salicylate (Levy, 1965). Throughout the duration of the trial no additional salicylates were permitted. However, because many arthritic patients cannot tolerate complete analgesic withdrawal, paracetamol and propoxyphene were made available to the patients throughout the trial. Neither of these compounds has been reported to interfere with the salicylate assays or salicylate metabolism (Amsal and Davidson, 1972; Thomas, Coldwell, Zeitz and Solomonraj, 1972).

The trial was blind in that neither the patients nor the physician knew which formulation was being administered. The order of administration of the various brands was determined by reference to a table of random numbers. A code was drawn up and held by a disinterested party until completion of the trial (Table IV).

Each of the patients (I-IV) in Table IV was required to take each of the five formulations (A - E) one each week, in the order 1 - 5. Thus each formulation was taken once by each patient and appeared a total of four times in this randomized block design. In practice, however, the trial

	Patients			
Formulations	I	II	III	IV
A	2	1	5	3
B	1	4	2	1
C	4	5	4	5
D	3	2	3	2
E	5	3	1	4

Table IV

Design of Trial

was modified to accommodate late patient admissions and early discharges from hospital. Four patients were available at the commencement of the trial. However, one patient could not tolerate salicylate withdrawal and another was discharged from hospital. One patient was admitted into hospital and subsequently into the trial in the third week.

The formulations selected were representative of those currently available in Canada. The dose of each preparation was 1.95 g ASA (six 5 gr tablets) administered as a single dose with water on an empty stomach on the morning of the test day. The patients were fasted overnight prior to dosing and received no food until noon of the test day (four hours after dosing). Significantly lower salicylate levels have been reported in non-fasting subjects (Spiers and Malone,

1967).

Trials were conducted on the same day each week for a total of five weeks. On the trial days the test dose was administered at approximately 8:00 a.m. Whole blood samples (7 ml) were drawn at 0, 1, 2, 4, 8 and 24 hours after administration. The blood samples were drawn into evacuated tubes containing heparin and fluoride as outlined. These were cooled on ice, centrifuged and the plasma pipetted off and stored on dry ice until required for analysis. Duplicate 1 ml portions of plasma were then assayed for SA and ASA content.

b) Plasma Concentrations

Tables V - VIII represent the plasma concentrations of each salicylate in each patient on each formulation. In these tables total salicylate is the sum of SA plus ASA following molar correction. Both SA and total salicylate are included because no corrections were made for ASA decomposition during storage.

The mean levels of each salicylate in all the patients on each formulation are depicted in Figures 15 - 19. Figure 20 compares the mean levels of total salicylate in all the patients on each of the five formulations. With the exception of the Bufferin curve (Figure 18) each experimental point represents the mean of three duplicate determinations from three patients. Four duplicate determinations constitute the points in the Bufferin curve.

Formulation	Hours After Dosing	μg SA per ml of Plasma	μg ASA per ml of Plasma	μg Total Salicylate per ml of Plasma
Aspirin	0	0	0	0
	1	45.0	11.8	53.3
	2	83.0	10.1	91.1
	4	109.0	0.7	115.3
	8	77.6	0	77.6
	24	73.1	0	73.1
Novasen	0	0	0	0
	1	5.6	0	5.6
	2	6.2	0	6.2
	4	18.4	5.9	22.6
	8	27.9	0	27.9
	24	11.0	0	11.0
Entrophen	0	0	0	0
	1	0	0	0
	2	0	0.9	0.7
	4	97.0	3.5	99.5
	8	100.0	0	100.0
	24	35.4	0	35.4
Bufferin	0	0	0	0
	1	115.6	0	115.6
	2	141.5	1.1	142.3
	4	124.7	16.3	136.2
	8	88.6	0	88.6
	24	15.9	0	15.9
Ecotrin	0	0	0	0
	1	0	0	0
	2	0	0	0
	4	39.7	6.7	44.4
	8	93.6	0.7	94.0
	24	33.3	0	33.3

Table V

Plasma Levels of Salicylates of Patient I

Formulation	Hours After Dosing	μg SA per ml of Plasma	μg ASA per ml of Plasma	μg Total Salicylate per ml of Plasma
Novasen	0	0	0	0
	1	4.1	0	4.1
	2	8.4	0	8.4
	4	141.1	3.8	143.8
	8	91.5	0	91.5
	24	1.0	0	1.0
Entrophen	0	0	0	0
	1	0	0	0
	2	0	0	0
	4	94.1	6.5	98.7
	8	82.8	0.8	83.4
	24	8.7	0	8.7
Bufferin	0	0	0	0
	1	90.1	18.1	102.8
	2	138.5	4.4	141.6
	4	132.7	0	132.7
	8	97.4	0	97.4
	24	6.7	0	6.7
Ecotrin	0	0	0	0
	1	0	0	0
	2	0	0	0
	4	34.0	3.7	36.6
	8	42.6	0.8	43.1
	24	19.0	0	19.0

Table VI

Plasma Levels of Salicylates of Patient II

Formulation	Hours After Dosing	μg SA per ml of Plasma	μg ASA per ml of Plasma	μg Total Salicylate per ml of Plasma
Aspirin	0	0	0	0
	1	110.8	14.0	121.2
	2	119.9	14.7	129.3
	4	160.8	1.0	161.5
	8	104.9	0	104.9
	24	0	0	0
Bufferin	0	0	0	0
	1	161.4	15.4	177.3
	2	77.5	0.9	78.1
	4	151.0	0	151.4
	8	111.6	0	111.6
	24	0	0	0

Table VII

Plasma Levels of Salicylates of Patient III

Formulation	Hours After Dosing	μg SA per ml of Plasma	μg ASA per ml of Plasma	μg Total Salicylate per ml of Plasma
Aspirin	0	0	0	0
	1	110.0	15.8	121.1
	2	107.0	3.1	109.2
	4	115.7	1.3	120.2
	8	79.5	0	79.5
	24	3.4	0	3.4
Novasen	0	0	0	0
	1	2.8	0	2.8
	2	30.6	5.7	34.7
	4	85.2	0	85.2
	8	30.7	0	30.7
	24	14.1	0	14.1
Entrophen	0	0	0	0
	1	0	0	0
	2	0	0	0
	4	35.2	5.8	39.2
	8	22.2	0	22.2
	24	38.6	0	38.6
Bufferin	0	0	0	0
	1	31.6	13.6	141.2
	2	153.2	0.8	153.8
	4	117.9	0	117.9
	8	73.0	0	73.0
	24	4.6	0	4.6
Ecotrin	0	0	0	0
	1	0	0	0
	2	0	0	0
	4	4.4	0	4.4
	8	4.6	0	4.6
	24	78.3	0	78.3

Table VIII

Plasma Levels of Salicylates of Patient IV

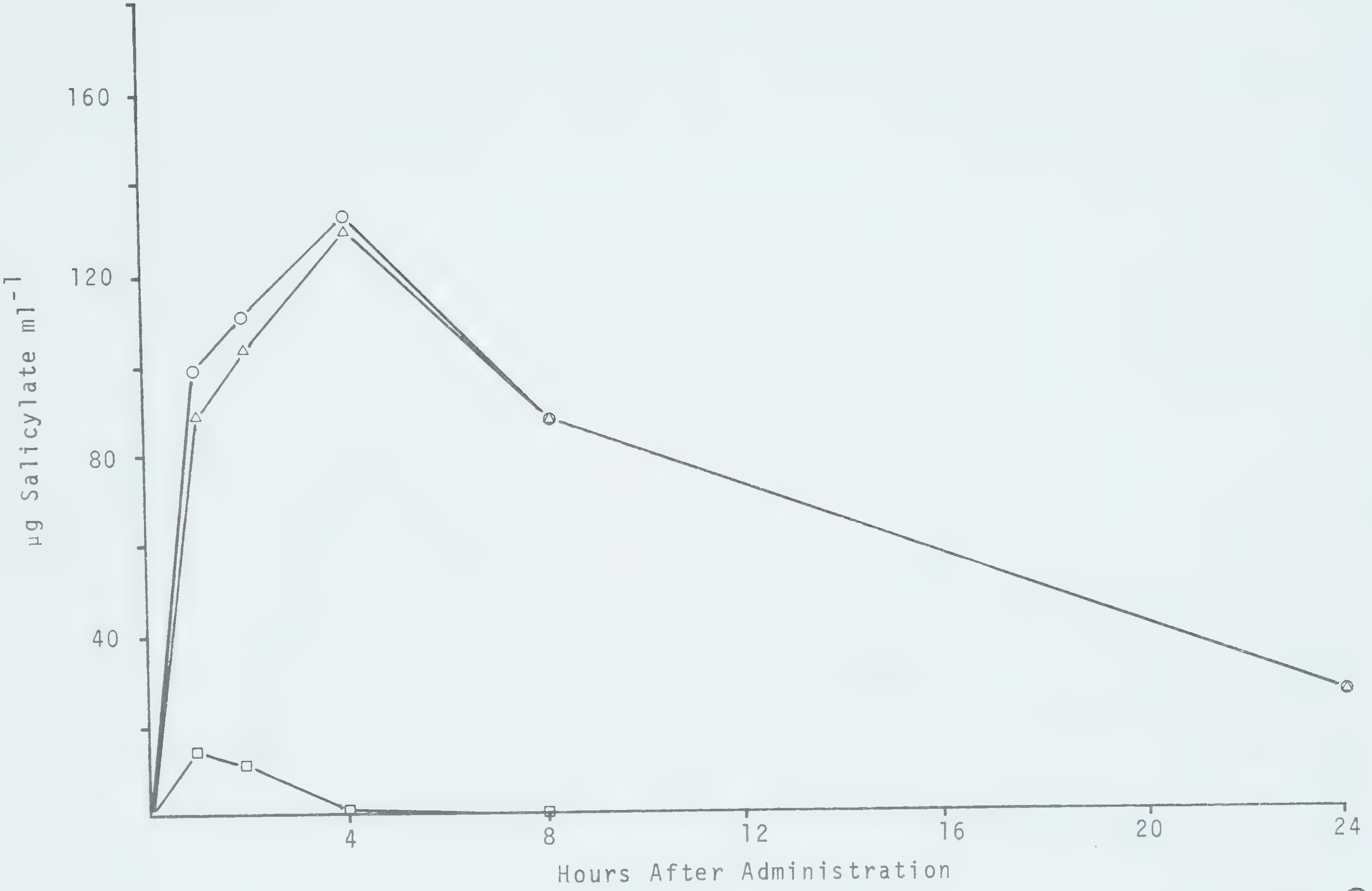


Figure 15: Mean Plasma Salicylate Levels of Patients on Formulation A Aspirin®

Key: ○————○ Total Salicylate
△————△ SA
□————□ ASA

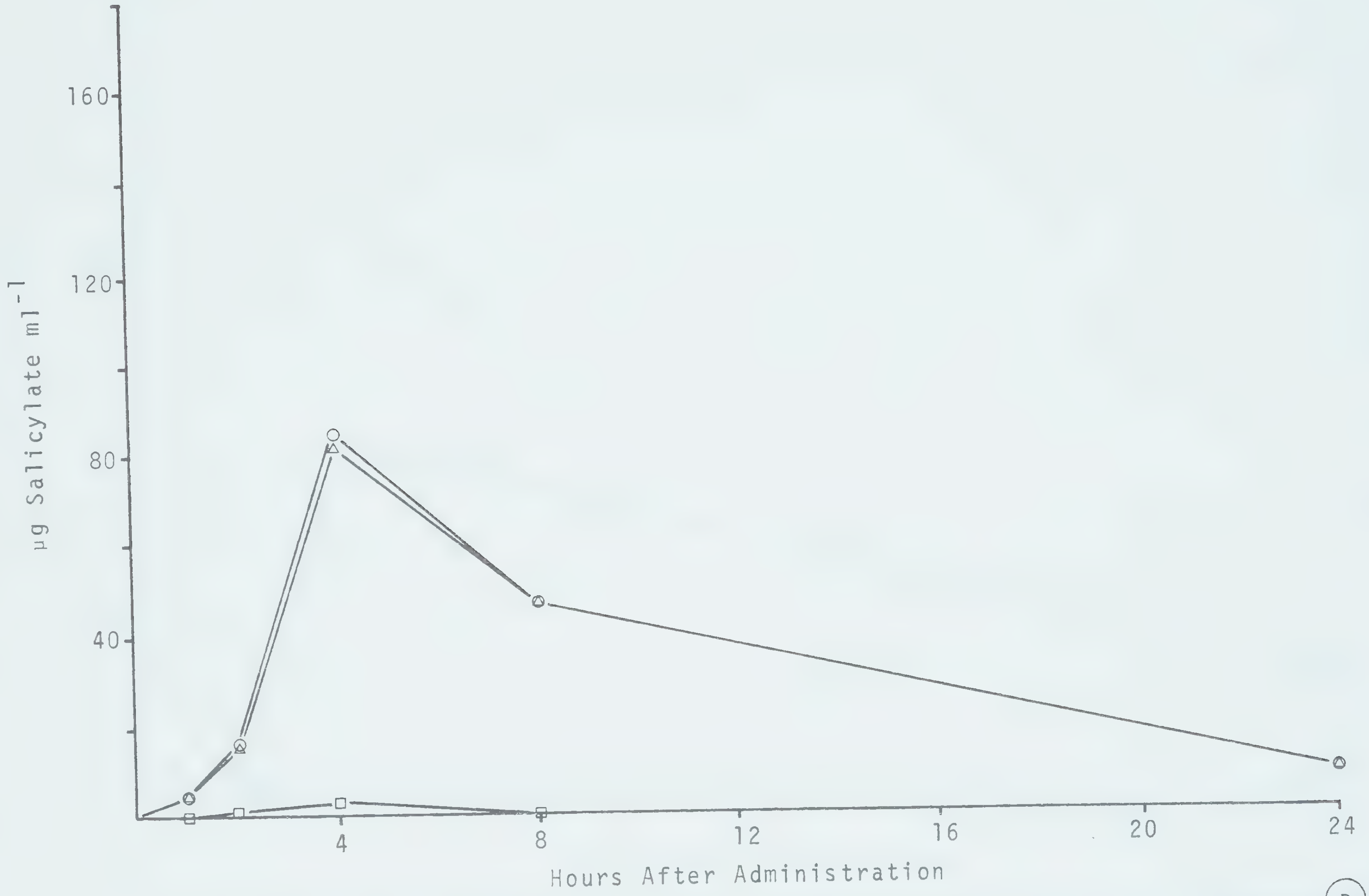


Figure 16: Mean Plasma Salicylate Levels of Patients on Formulation B Novasen ®

Key: ○———○ Total Salicylate
△———△ SA
□———□ ASA

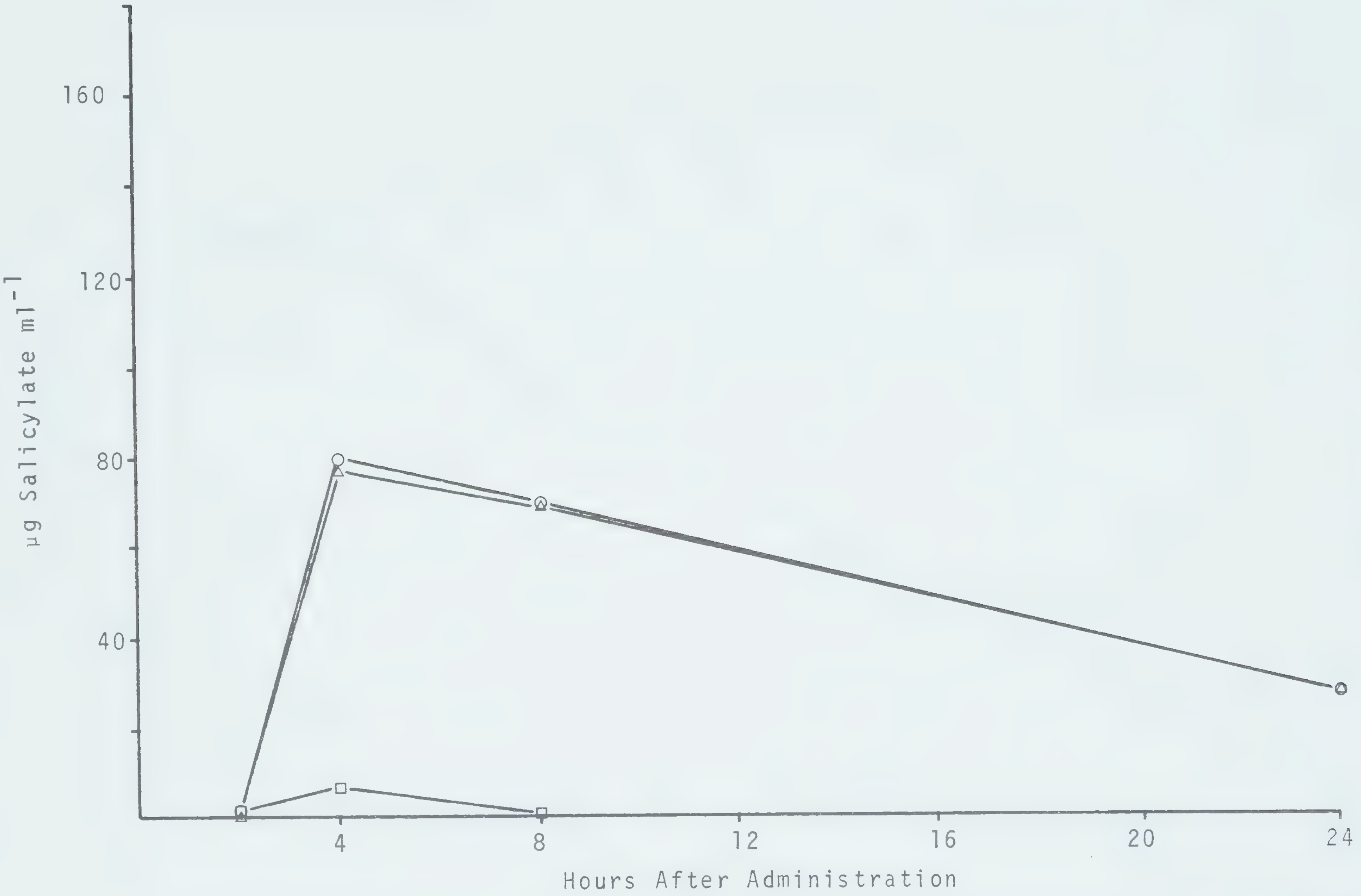


Figure 17: Mean Plasma Salicylate Levels of Patients on Formulation C Entrophen®

Key: ○———○ Total Salicylate
△———△ SA
□———□ ASA

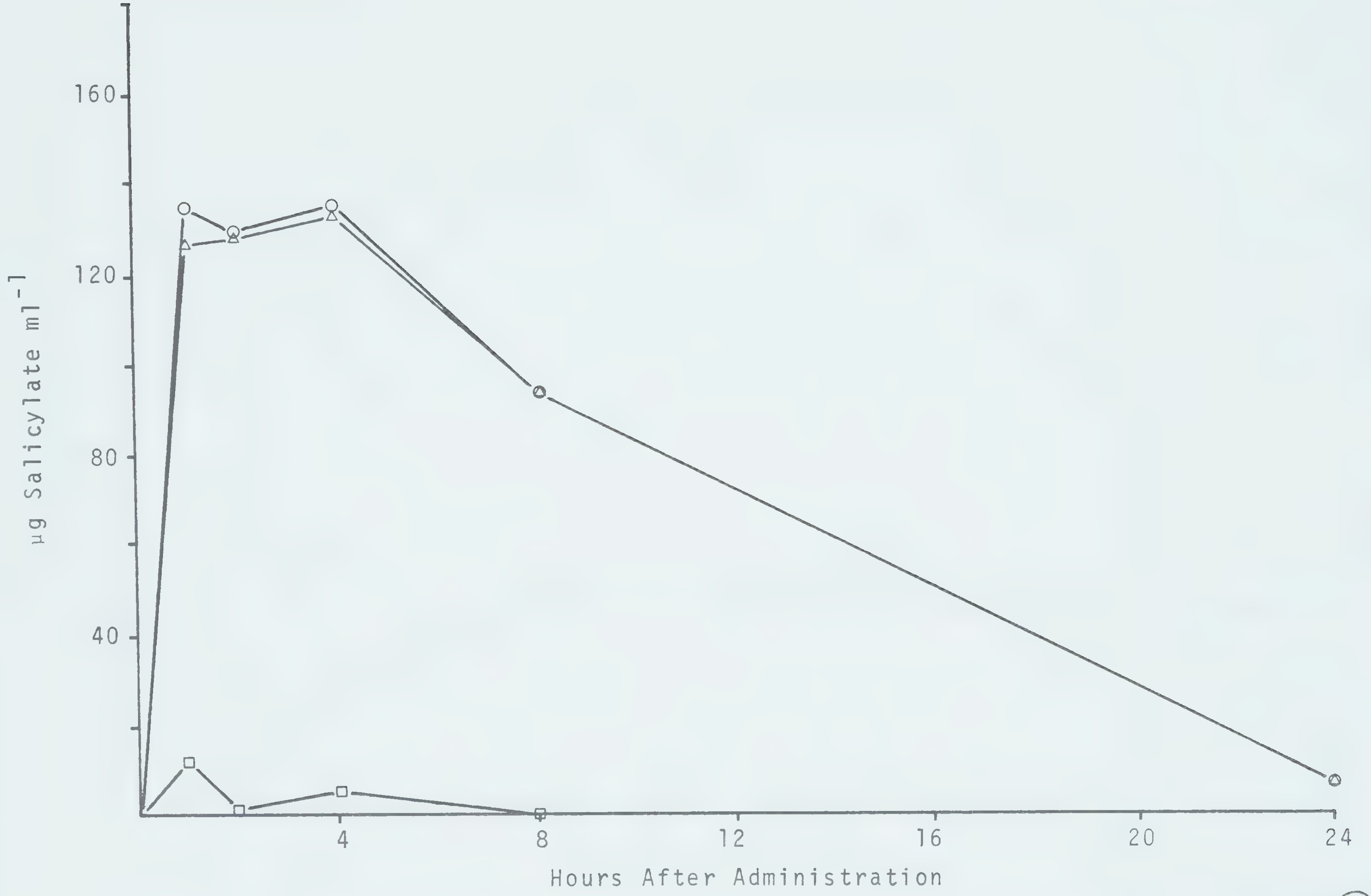


Figure 18: Mean Plasma Salicylate Levels of Patients on Formulation D Bufferin®

Key: ○———○ Total Salicylate
△———△ SA
□———□ ASA

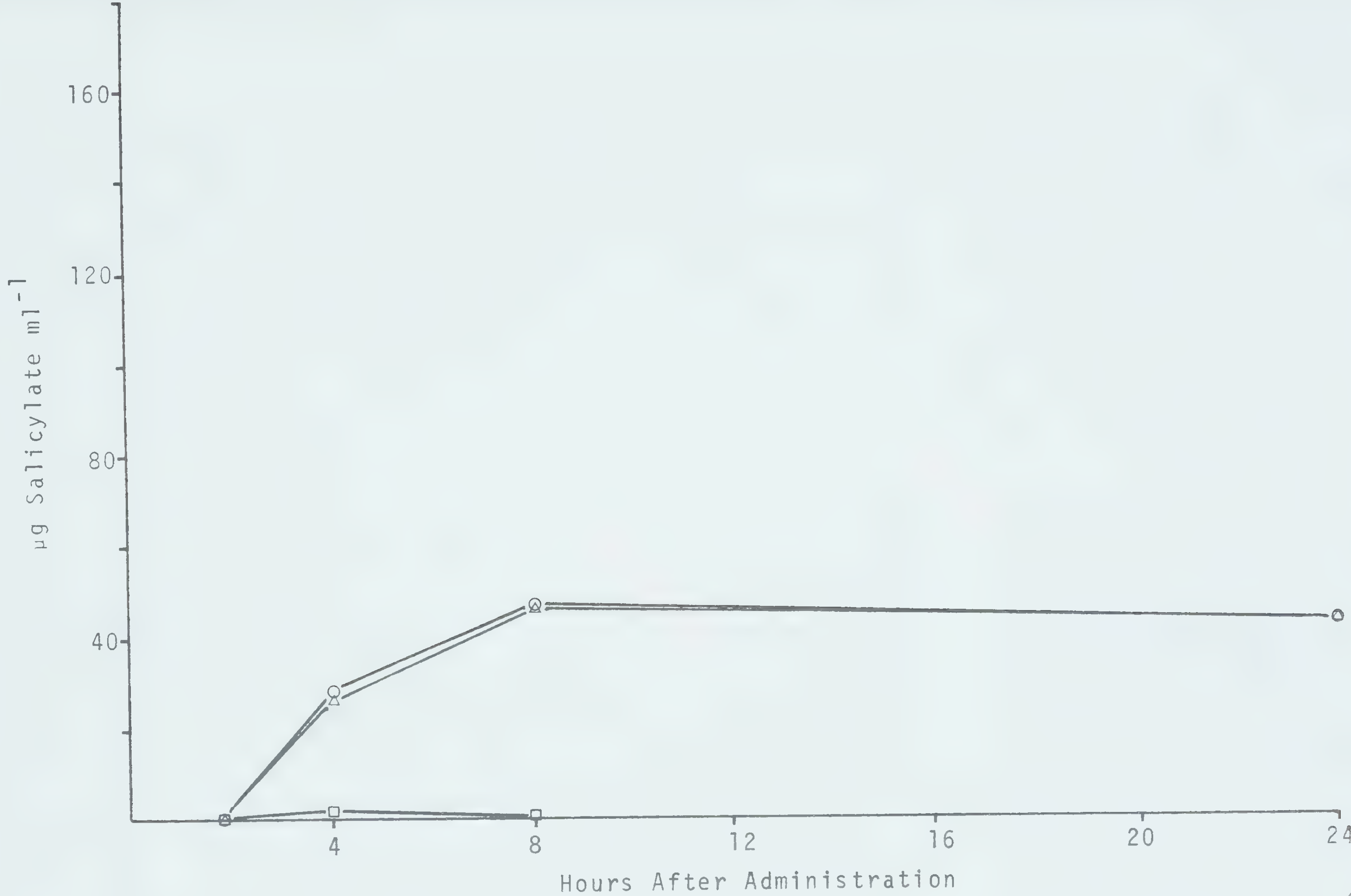


Figure 19: Mean Plasma Salicylate Levels of Patients on Formulation E Ecotrin®

Key: ○———○ Total Salicylate

△———△ SA

□———□ ASA

The data in Figure 20 suggested that there was essentially no difference in the rate of absorption of Aspirin versus Bufferin, although the latter formulation appeared to produce slightly higher plasma levels. Bufferin seemed to have a shorter half-life than Aspirin but the difference in mean plasma total salicylate levels was not found to be statistically significant.

c) Bioavailability and Disintegration Data

Table IX expresses the data from Figure 20 in tabular form. The Four Hour Availability histogram compares the bioavailability of each formulation at four hours with its total availability over 24 hours. Four hours was selected as the time for comparison as it corresponds to the peak plasma levels of most of the formulations. The measure of bioavailability used in these studies was the area under the respective plasma total salicylate curve (Figure 20). The Four Hour histogram indicated that Bufferin was slightly more available than Aspirin in four hours. The Twenty-four Hour histogram compares the availability of each formulation over 24 hours to that of Aspirin over 24 hours. The difference observed between the Bufferin and Aspirin availability at four hours is minimized over 24 hours.

Numerous papers have appeared in the biopharmaceutical literature concerning the correlation of *in vitro* tests and pharmacological availability of various drug preparations. The ultimate aim of such studies is the development of a

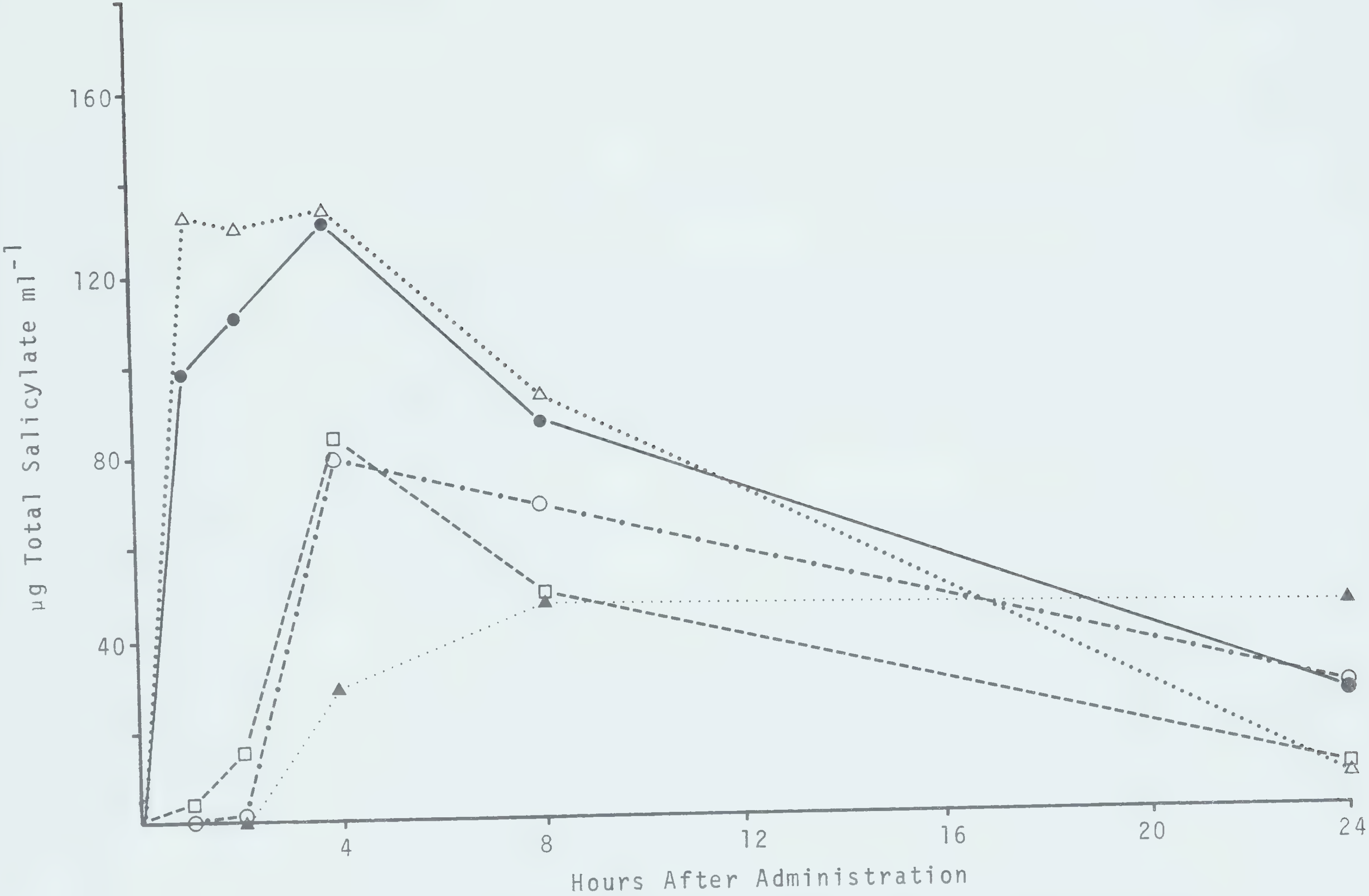


Figure 20: Comparison of Mean Plasma Total Salicylate Levels

Key: △··········△ Bufferin
●————● Aspirin
□- - - - - -□ Novasen
○-·-·-·-○ Entrophen
▲··········▲ Ecotrin

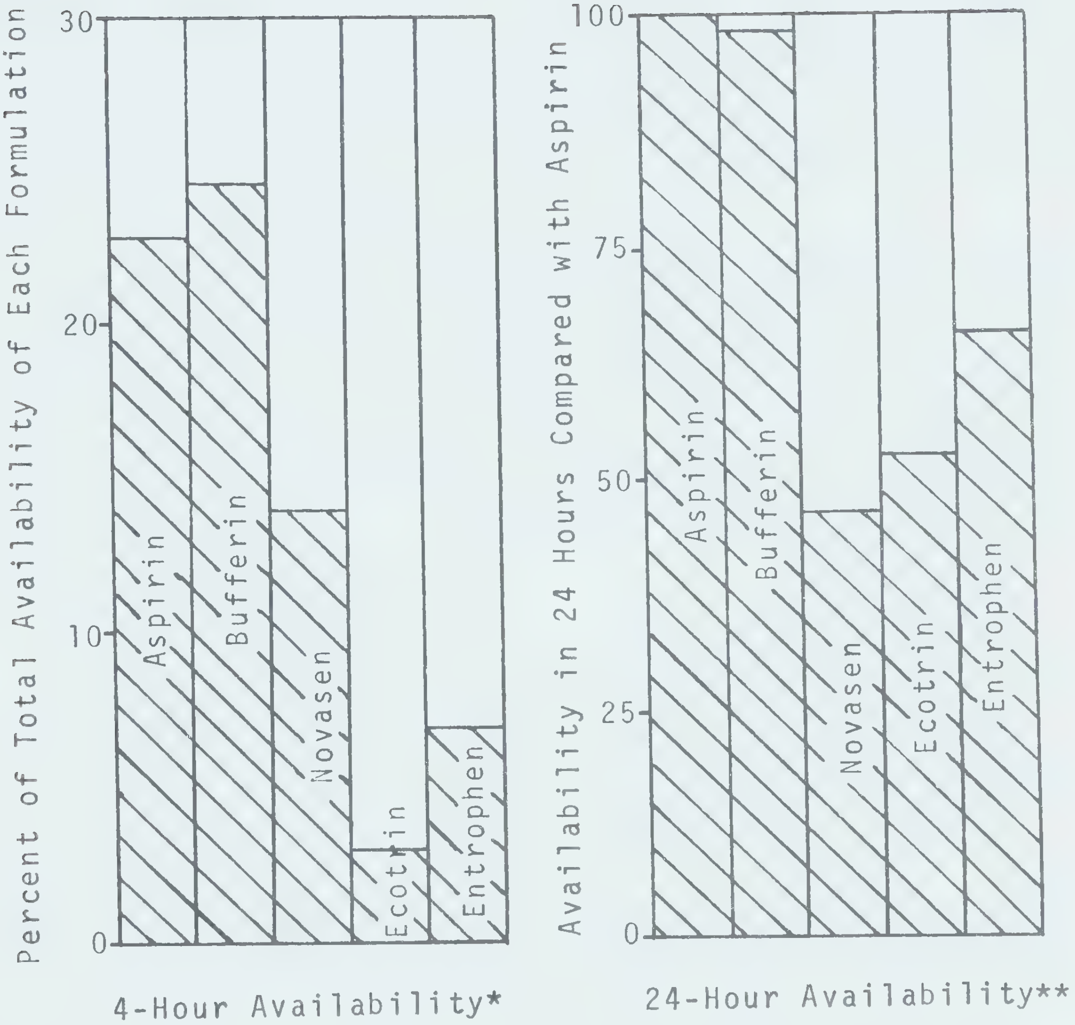


Table 9

Bioavailability of Various Formulations
of Acetylsalicylic Acid
at Four and Twenty-four Hours

* Bioavailability of the preparation in 4 hours expressed as a percent of total bioavailability observed over 24 hours

** Bioavailability of each preparation observed over 24 hours compared to the bioavailability of Aspirin

simple *in vitro* test which will enable the prediction of the more complex properties of a drug in a biological system. This predictability would be useful for quality control and compendial purposes as well. It has been stated that the best model of any system is the system itself (Swarbrick, 1970), but the *in vitro* tests have obvious advantages in terms of cost and manpower.

It has been shown (Levy, 1964) by a successful correlation of *in vitro* and *in vivo* data that dissolution is the rate-limiting step in the oral absorption of solid dosage forms of ASA. Disintegration of solid formulations of ASA precedes the dissolution and may interfere with the absorption of ASA by delaying the dissolution rate (Levy, 1960).

With this information concerning bioavailability in hand, an *in vitro* method of bioavailability determination, which could be correlated with this *in vivo* data was sought. The *in vitro* test selected was the USP Disintegration Test. Six tablets of each formulation, from the same lots as used in the *in vivo* determinations, were subjected to the Disintegration Test, using each of the two fluids, Simulated Gastric Fluid, T.S. (pH 1.2), and Simulated Intestinal Fluid, T.S. (pH 7.5). The percent availability of each formulation in four hours to Aspirin is included with the disintegration data in Table X. As expected, none of the enteric-coated preparations disintegrated in the Gastric Fluid within two hours. It is significant to note that among the enteric-

Formulation	Seconds to Disintegrate in Simulated Gastric Fluid T.S.	Seconds to Disintegrate in Simulated Intestinal Fluid T.S.	Percent Available in Four Hours Compared to Aspirin
Aspirin	17	12	100
Bufferin	20	21	118
Novasen	--*	885	31
Entrophen	--*	995	21
Ecotrin	--*	1043	7

* no disintegration in 2 hours

Table X

Correlation of Bioavailability with Disintegration Time in USP Disintegration Tests

coated preparations there was a correlation of the time required to disintegrate in Intestinal Fluid, and the percent availability in four hours. Thus Novasen disintegrated fastest (885 sec) and was most available (31%) while Ecotrin was the least available in four hours (7%) and required the longest time to disintegrate (1043 sec). Numerous authors have previously presented data to support a similar correlation of disintegration and absorption rates in healthy human subjects (Levy, 1960; Levy, Leonards and Procknall, 1965; Rasmussen, 1968; Gibaldi and Weintraub, 1970). However, little data is available in the literature regarding such a relationship in arthritic patients.

While rapid disintegration of uncoated ASA tablets has been equated with rapid drug absorption, such is not always the case. It has been observed in one study (Levy, 1960) that the faster absorbed formulations of ASA had longer disintegration times than did the more slowly absorbed products. With enteric-coated products, on the other hand, disintegration time may be one of the determining factors in absorption (Rasmussen, 1968).

The half-lives of SA in each formulation were calculated using the mean plasma levels of total salicylate at 8 and 24 hours from the data in Figure 20. The values obtained were: Aspirin, 9.60 hr; Novasen, 6.08 hr;

Entrophen, 11.74 hr; Bufferin, 4.08 hr; and Ecotrin, 123.75 hr.* With the exception of the Ecotrin, this data compares favourably with the half-lives quoted earlier in this thesis (Brodie, Burns and Weiner, 1959; Swintosky, 1956).

Both uncoated formulations were absorbed at a much higher rate and to a greater extent than were the three enteric-coated brands. Among the enterics, Ecotrin and Entrophen exhibited a lag period of approximately two hours before significant absorption occured, while Novasen showed substantial plasma salicylate levels within two hours. Novasen and Entrophen produced comparable plasma salicylate levels while Ecotrin resulted in somewhat lower but more sustained plasma salicylate concentrations.

One method of expressing drug blood levels in terms of absorption is to calculate the percent absorbed versus time (Wagner and Nelson, 1963) using the following equation:

$$\frac{A_\tau}{V} = C_\tau + K\int_{t=0}^{t=T} C\,dt.$$

Here A_τ is the amount of drug absorbed from time of administration to time T, V is the apparent volume of distribution, C_τ is the blood level of drug at time T, K is the first order rate constant for loss of drug from the volume of distribution and C is the blood level in concentration units at

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* The exceptionally long mean half-life for Ecotrin is in large part due to an unusually long lag phase preceeding absorption in patient IV.

time T. Briefly, the method involves calculation of the areas under the plasma concentration-time curve (Figure 20) in one-hour increments (0-1 hr, 1-2 hr, etc.). These areas are multiplied by the appropriate elimination rate constant (determined for each formulation) and the product is added to the respective plasma level. The resultant values, representing plasma levels corrected for excretion, progressively increase until an asymptotic value is reached. When the individual values are expressed as a percentage of the maximum, the results are percent absorbed versus various times (Table XI). This method assumes that V and K remain constant and that the drug is completely absorbed during the period of investigation.

Formulation	Time Range (hours after administration)							
	0-1	1-2	2-3	3-4	4-5	5-6	6-7	7-8
Aspirin	69.2	83.8	91.4	100.0	100.0	100.0	100.0	100.0
Novasen	4.6	18.6	57.7	100.0	100.0	100.0	100.0	100.0
Entrophen	0	0	49.0	100.0	100.0	100.0	100.0	100.0
Bufferin	85.1	96.5	98.0	100.0	100.0	100.0	100.0	100.0
Ecotrin	0	0	30.0	60.3	100.0	100.0	100.0	100.0

Table XI

Percent Absorbed Versus Time

The results at one hr for Aspirin (69.2%) and for Bufferin (85.1%) agree fairly well with previously published data (Levy, 1964) which quotes 68.0% for plain ASA and 84.0% for buffered ASA.

The percent absorbed versus time data suggested that among the enteric-coated preparations the rate of absorption was Novasen > Entrophen > Ecotrin. Also Bufferin appeared to be absorbed somewhat faster than Aspirin.

The results of these in vivo and in vitro bioavailability determinations suggested that the most bioavailable enteric-coated preparation of the formulations tested was Novasen. This preparation had the highest percentage absorption versus time, was the most available in four hours, and was not subject to an initial lag phase preceding absorption. However, it is clear that all three enteric-coated preparations, properly used, could give adequate salicylate levels in rheumatoid patients.

However, there are numerous difficulties associated with bioavailability trials in humans. First of all, the urine pH is expected to affect the excretion rate of free salicylate (Goodman and Gilman, 1970; Levy and Leonards, 1971). A second difficulty is the control of gastric motility. The rate of gastric emptying is expected to influence the passage of drugs into the small intestine and their subsequent abospriton (Leonards and Levy, 1965). This factor is significant in enteric-coated preparations which are not absorbed at all from the stomach. The

presence of food in the stomach and the volume of water with which the drug is taken both affect gastric emptying (Hunt, 1963). Thus despite the care taken during the study to eliminate these sources of error, the conclusions of the study should be regarded in a comparative rather than an absolute context.

II. Salicylate Ascorbic Acid Interactions

a) Design of Trial

The reported interaction of ascorbic acid with salicylate absorption (Staudacher and Müller, 1969) was selected for further investigation in the following study. The objectives of the experiment were twofold, namely:

1) to determine whether ascorbic acid, administered orally, affects concommitant salicylate absorption, and
2) to determine whether acetylsalicylic acid, administered orally, affects the simultaneous oral absorption of ascorbic acid.

The trial was designed as follows: Female New Zealand White rabbits of approximately 2.5 kg weight were selected as the experimental animals. Rabbits have been reported to have a serum ASA hydrolysis rate comparable to the human rate (Truitt and Morgan, 1965), and are relatively easy to obtain serial blood samples from. They were starved for 24 hours prior to dosing and food was withheld for the duration of the trial. Water was permitted ad libitum. The animals were randomly assigned to receive one of three possible doses:

1. 320 mg ASA
2. 200 mg ascorbic acid
3. 320 mg ASA plus 200 mg ascorbic acid

The drugs were finely divided and suspended in 1% aqueous tragacanth immediately prior to dosing. The final concentration of each suspension was such that a 2 ml aliquot represented a dose.

Whole blood samples (2 ml) were obtained at 0, 1, 2, 4 and 8 hours after administration of the drugs. The blood samples were drawn into syringes containing 10 units of heparin and 2.5 mg KF in 15 µl of water (free of preservatives). This blood was cooled, centrifuged and the plasma pipetted off and stored frozen on dry ice until required for analysis. The plasma was then thawed and duplicate 0.3 ml portions were analyzed for salicylate as described previously. The amount of ASA found in these samples (both following ASA alone as well as after ASA plus ascorbic acid) was consistently low and never exceeded 0.5 µg 0.3 ml^{-1}. Because the contribution of ASA to total salicylate was insignificant it was ignored in this experiment. Again positive controls were employed with each group of unknown samples assayed.

Plasma ascorbic acid was determined colorimetrically by a method which involves reaction with 2,4-dinitrophenylhydrazine (Natelson, 1971). Separate 0.2 ml portions of plasma were assayed for ascorbic acid.

It was shown that salicylate and acetylsalicylate did not interfere with the ascorbic acid assay and conversely, ascorbic acid did not interfere with the salicylate assay.

Ideally, experimental animals should be dosed on a mg of drug per kg of body weight basis. In this trial, however, it was decided to dose with a standard weight of drug per animal to allow for possible further experimentation with enteric-coated preparations, which are available commercially only in formulations of 320 and 640 mg of ASA.

The serial blood samples were drawn from the marginal ear veins of the animals. It was observed that as little as five samples drawn successfully during a single trial damaged these veins to the point that the animals could not be used again. Consequently, the rabbits were destroyed after a single run and a cross-over feature could not be incorporated into this trial.

In view of these two limitations the following corrections were made:

1. Each observed plasma salicylate level in groups 1 and 3 was corrected on a weight basis to correspond to that expected for a 2.5 kg animal. This is justified theoretically at least, in that a small animal would be expected to have a higher plasma level of a drug than would a large animal.
2. The initial (zero hour) ascorbic acid levels in groups 2 and 3 were subtracted from the corresponding 1, 2, 4 and 8 hour ascorbic acid levels. The resultant figures

were then corrected, again on a weight basis to correspond to that expected for a 2.5 kg animal.

b) Plasma Salicylate Concentrations

Table XII represents the corrected plasma concentrations of salicylic acid in the four animals which received ASA alone. Similarly, Table XIII depicts the SA plasma levels in the four animals which received ASA plus ascorbic acid. The mean salicylate concentrations in these two test groups are compared in Figure 21.

c) Plasma Ascorbic Acid Concentrations

The corrected plasma ascorbic acid levels of the six animals which received ascorbic acid alone are given in Table XIV. Table XV represents the ascorbic acid levels of the seven rabbits which received ascorbic acid plus ASA. A comparison of the mean ascorbic acid levels of these two groups is given in Figure 22.

d) Statistical Evaluation of Interaction

Inspection of Figure 21 suggested the possibility that the presence of ascorbic acid enhances the oral absorption of salicylate. These means, at 1, 2, 4 and 8 hours were compared statistically by Students 't' test. The values obtained were 1.13 (1 hr), 1.37 (2 hr), 1.17 (4 hr) and 0.93 (8 hr). With six degrees of freedom, the value of t required for significance ($p=.05$) is 2.45. Consequently, none of the apparent differences between the four pairs of

		µg SA per 0.3 ml Plasma				
Animal	Weight (kg)	0 hr	1 hr	2 hr	4 hr	8 hr
3	3.10	0	40.9	40.3	36.1	15.9
5	2.23	0	34.7	52.2	62.7	40.0
7	2.20	0	25.4	37.4	41.4	40.0
10	2.48	0	48.7	41.4	41.9	40.1

Table XII

Corrected Plasma Salicylate Levels After 320 mg ASA

		µg SA per 0.3 ml Plasma				
Animal	Weight (kg)	0 hr	1 hr	2 hr	4 hr	8 hr
2	3.10	0	48.6	66.6	87.2	78.0
4	2.72	0	50.0	53.9	42.2	20.1
8	2.12	0	33.9	39.9	47.1	34.9
9	2.37	0	44.8	46.9	59.7	54.4

Table XIII

Corrected Plasma Salicylate Levels After 320 mg ASA
Plus 200 mg Ascorbic Acid

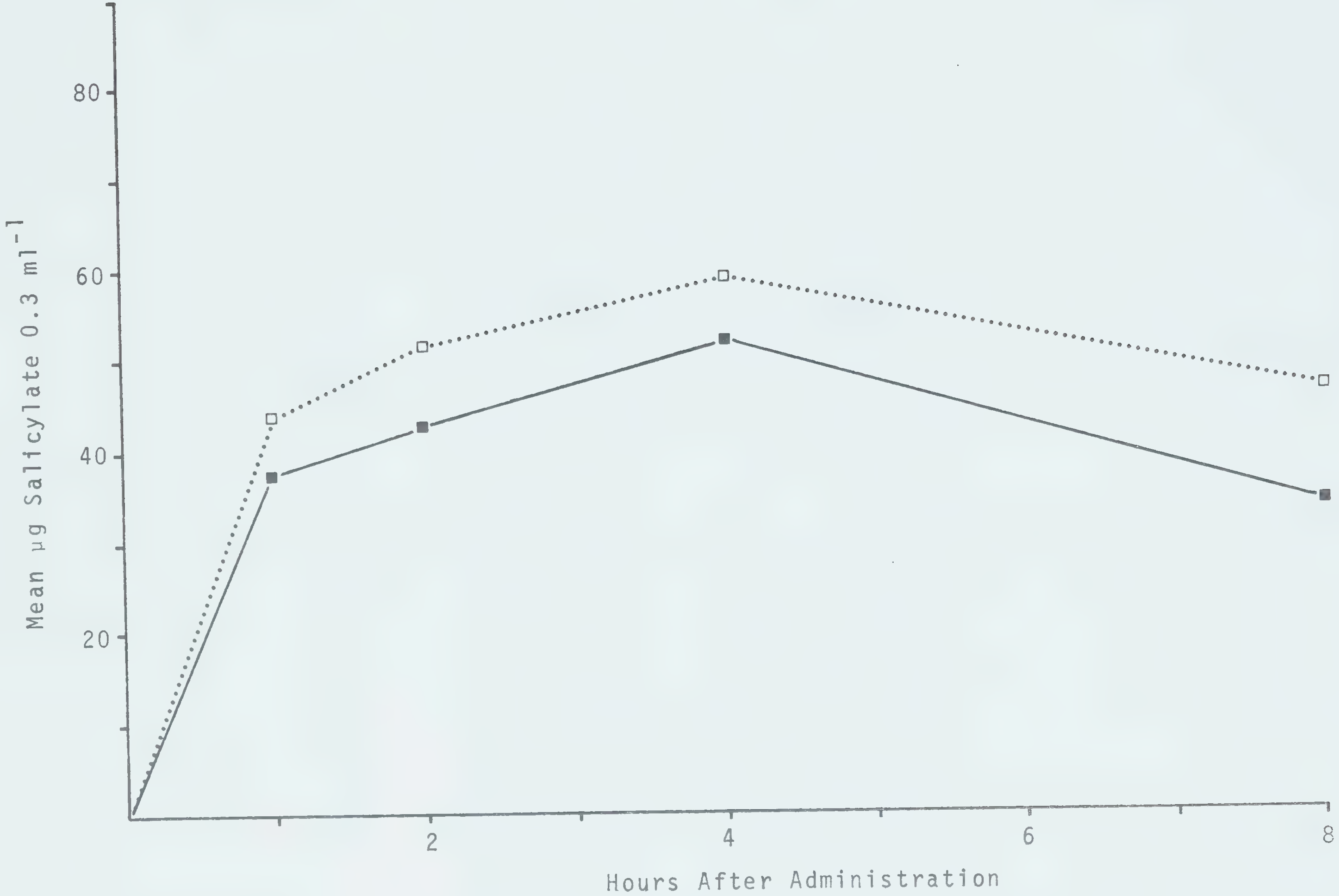

Figure 21: Corrected Mean Plasma Salicylate Levels After 320 mg ASA and After 320 mg ASA Plus 200 mg Ascorbic Acid

Key: □⋯⋯⋯□ ASA Plus Ascorbic Acid

■——■ ASA

		mg Ascorbic Acid per 100 ml Plasma				
Animal	Weight (kg)	0 hr	1 hr	2 hr	4 hr	8 hr
1	3.19	0	1.39	2.53	2.03	0.91
6	3.46	0	0.64	2.05	3.32	1.33
11	2.30	0	0.33	0.72	2.18	1.18
12	2.53	0	0.29	1.09	1.87	0.59
14	2.15	0	0.24	0.58	1.02	1.01
15	2.34]	0	0.80	1.54	2.12	0.88

Table XIV

Corrected Plasma Ascorbic Acid Levels
After 200 mg Ascorbic Acid

		mg Ascorbic Acid per 100 ml Plasma				
Animal	Weight (kg)	0 hr	1 hr	2 hr	4 hr	8 hr
2	3.10	0	0.69	1.77	3.30	4.20
4	2.72	0	1.57	3.10	2.68	2.18
8	2.12	0	1.98	2.85	3.66	2.54
9	2.37	0	1.89	2.73	3.09	2.25
13	2.10	0	0.22	0.44	0.81	0.42
16	2.27	0	0.39	1.34	1.55	1.06
17	2.15	0	1.07	1.46	3.41	2.41

Table XV

Corrected Plasma Ascorbic Acid Levels
After 200 mg Ascorbic Acid Plus 320 mg ASA

means was statistically significant. Thus whilst a consistent difference was observed, it was not statistically significant. Clearly more animals are needed. These results are interpreted as evidence to suggest that the administration of ascorbic acid had no effect on the concommitant absorption of salicylate, at least in this experiment.

Figure 22 suggested that the presence of ASA produced an increase in the total absorption of ascorbic acid. Students 't' test was also applied to these means, and the resultant values of t were: 1.56 (1 hr), 0.81 (2 hr), 1.08 (4 hr) and 2.52 (8 hr). The minimal value of t required for significance (p=.05) for 11 degrees of freedom is 2.20. Consequently, the pairs of means at 1, 2 and 4 hours were not statistically different, while the pair at 8 hours showed a significant difference. The results of this experiment suggested that ASA did not produce a statistically significant increase in the absorption of ascorbic acid. The observed difference at 8 hours occurred after the absorptive phase was completed. One explanation of the observed difference is that ascorbic acid and salicylic acid compete for renal excretion. However, a more adequate explanation of the difference may be related to fluctuations of urinary pH. For example, the mean rate of salicylate clearance is approximately four times as great at pH 8.0 than at pH 6.0 (Goodman and Gilman, 1970). A decrease in urinary pH from pH 6.5 to 5.5 in a patient on a dosage regimen designed to

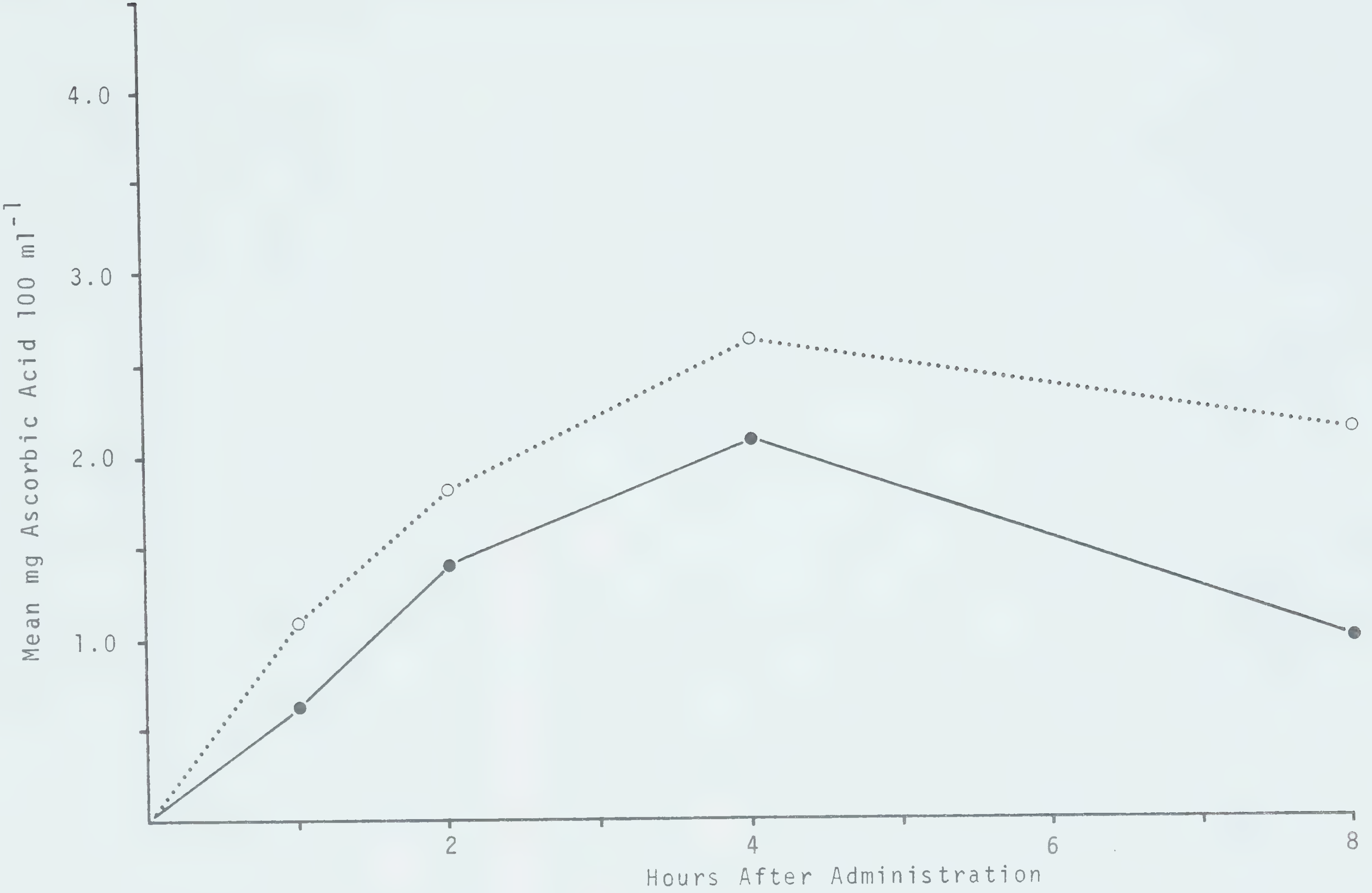

Figure 22: Corrected Mean Plasma Ascorbic Acid Levels After 200 mg Ascorbic Acid and After 200 mg Ascorbic Acid Plus 320 mg ASA

Key: ○·············○ Ascorbic Acid Plus ASA

●————● Ascorbic Acid

produce approximately 250 μg salicylate ml^{-1} is likely to produce plasma concentrations twice as high (Levy and Leonards, 1971). Unfortunately, urinary pH was not controlled or monitored in this trial.

The results obtained in this rabbit trial disagree with those obtained in humans (Staudacher and Müller, 1969). Several factors may be involved in this discrepancy. First of all, it has been reported that the stomachs of New Zealand White rabbits which have been starved for 24 hours are nearly as full as those of unfasted rabbits, and that significant material remained in the stomachs after one week of fasting (Chiou, Riegelman and Amberg, 1969). It has therefore been concluded that rabbits' stomachs emptied very slowly in the fasted state. Both the volume and the nature of these stomach contents have obvious implications on concommitant drug absorption.

A second factor to consider is that of different formulations used in the human trial. ASA was administered in plain tablets while the combination tablets of ascorbic acid and acetylsalicylic acid were described as containing 'water-soluble acetylsalicylic acid'. An increase in the rate of dissolution of a drug in the gastrointestinal fluids would be anticipated to result in faster absorption and higher initial plasma levels.

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